RETENTION OF THE HARPAGOSIDE CONTENT IN DRIED *HARPAGOPHYTUM PROCUMBENS* (DEVIL'S CLAW) ROOT THROUGH CONTROLLED DRYING AND THE APPLICATION OF NEAR INFRARED SPECTROSCOPY (NIRS) AS RAPID METHOD OF DETERMINATION

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I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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ABSTRACT

The effect of drying conditions on the retention of harpagoside, a glucoside iridoid found in the indigenous Southern African plant *Harpagophytum procumbens* (Devil's Claw), was investigated. Additionally, the feasibility of using near infrared spectroscopy (NIRS) as a rapid and non-destructive analytical tool for the prediction of the iridoids harpagoside (HS), 8-ρ-coumaroyl harpagide (8ρCHG) and moisture contents, as well as the HS:8ρCHG ratio in Devil's Claw was also studied.

Due to the purported medicinal activity of the tuberous secondary roots, Devil's Claw is of commercial interest with a well established export market in Germany. The stability of HS during processing has, however, never been investigated and the current study evaluated the effect of three drying methods (sun, tunnel and freeze-drying), as well as three tunnel-drying temperatures (40°C, 50°C and 60°C, dry bulb temperature) on HS content.

Methanolic extracts (70% methanol-water) were analysed by high-performance liquid chromatography (HPLC) and a multiple extraction test (n = 12) indicated a 91.70% recovery for HS. The comparative drying results showed significant ($P < 0.05$) differences between tunnel and freeze drying on the one hand and sun-drying on the other, with the latter being the most detrimental technique. In comparing tunnel-drying temperatures, at a constant relative humidity (RH) of 30%, drying at 40°C showed the lowest retention of HS, differing significantly ($P < 0.05$) from that at 50°C. Drying at 60°C did not differ significantly ($P > 0.05$) from either of the other two conditions, but also resulted in lower retentions of HS content than drying at 50°C.

Overall, freeze-drying showed excellent results for HS retention (1.565% ± 0.394%; mean ± standard deviation), but economic constraints limit the commercial application of this drying method. Although further study is required to optimise tunnel-drying conditions, this method indicated acceptable and practical results (1.526% ± 0.396%), especially with conditions set to 50°C and 30% RH (1.750% ± 0.438%). Future research should concentrate on elucidating the degradation mechanisms of HS during processing, transport and storage of dried Devil's Claw root.

The analysis of dried, ground Devil's Claw root by NIRS was referenced to HPLC analyses of the same methanolic extracts used in the drying studies. The spectra were generated on two different NIRS instruments by measuring diffuse reflectance of the powder whilst calibrations were performed by partial least squares regression. Both independent data set validation and full cross-validation were used to evaluate the performance and predictive abilities of the various models. Excellent moisture content (MC) calibrations were developed with a

standard error of prediction (SEP) of 0.24% (range: 2.44% to 10.43%) and correlation coefficient (*r*) of 0.99. For the given MC range, the model performed very well against the standard error of laboratory (SEL) of 0.14%.

Considering the sensitivity of the HPLC reference method (SEL = 0.035%), the HS content calibration (range: 0.693% to 2.244%) performed admirably with an SEP of 0.134%. The available range was, however, relatively small and future NIRS studies should incorporate a larger range if possible. The 8ρCHG model (range: 0.069% to 0.290%) performed similarly, with a standard error of cross-validation of 0.028% and SEL of 0.007%.

By inclusion of a second species, *H. zeyheri*, both the 8ρCHG content and especially HS:8ρCHG ratio (range: 1.84 to 34.48) calibrations were evaluated as possible methods of distinguishing between species. With 8ρCHG values of *ca.* 0.9%, good separation was achieved for the first model but, contrary to previous studies, the ratio was found to be less successful. Principle component analysis of the spectra, however, showed great potential as a qualitative tool for this purpose.

It is believed that NIRS can become an invaluable tool for the division of Devil's Claw into export classes, depending on HS content. This content is, however, dependant on the drying conditions used and drying parameters should be optimised to ensure the best possible quality and retention of HS.

UITTREKSEL

Die invloed van drogingskondisies op die behoud van harpagosied, 'n iridoïed glikosied wat in die inheemse Suider-Afrikaanse plant, *Harpagophytum procumbens* (Duiwelsklou) voorkom, is ondersoek. Die uitvoerbaarheid van naby infra-rooi spektroskopie (NIRS) as 'n vinnige en nie-destruktiewe analitiese metode vir die voorspelling van die inhoud van iridoïede, harpagosied (HS),

8-ρ-kumaroïel-harpagied (8ρCHG) en vog, sowel as die HS:8ρCHG verhouding in Duiwelsklou is ook ondersoek.

Die beweerde medisinale aktiwiteit van die knolagtige sekondêre wortels van Duiwelsklou het gelei tot 'n gevestigde uitvoermark in Duitsland, en is dus van kommersiële belang. Die stabiliteit van HS gedurende prosessering is nog nooit ondersoek nie. Die huidige studie het die invloed van drie drogingsmetodes (son-, tonnel- en vriesdroging) en drie tonneldrogingstemperature (40°C, 50°C en 60°C, droëbol temperatuur) op die HS inhoud evalueer.

Metanolies ekstrakte (70% metanol-water) is met hoë-druk vloeistof chromatografie ("HPLC") geanaliseer en 'n veelvoudige ekstraksie toets (n =12) het 'n 91.70% herwinning van HS getoon. Betekenisvolle ($P < 0.05$) verskille is gevind tussen tonnel- en vriesdroging enersyds en sondroging andersyds, met laasgenoemde die nadeligste metode. Die vergelyking van tonneldrogingstemperature, by 'n konstante relatiewe humiditeit (RH) van 30%, het getoon dat droging by 40°C die minste HS behou het en betekenisvol ($P < 0.05$) verskil het van droging by 50°C. Alhoewel die droging by 60°C nie betekenisvol verskil het van die ander twee kondisies nie, het dit laer HS waardes as droging by 50°C getoon.

Vriesdroging het uitstekende resultate ten opsigte van die behoud van HS gelewer (1.565% ± 0.394%; gemiddelde ± standaard afwyking), maar ekonomiese beperkings plaas noodgedwonge limiete op die toepaslikheid van hierdie drogingsmetode. Verdere studie om tonneldrogingskondisies te optimiseer word is nodig (1.526% ± 0.396%), maar veral droging by 50°C en 30% RH (1.750% ± 0.438%) het prakties aanvaarbare resultate gelewer. Toekomstige navorsing moet daarop gemik word om die spesifieke degradasie meganismes van HS gedurende die droging, vervoer en opberging van Duiwelsklouwortel te verklaar.

Die NIRS ontleding van gedroogde, gemaalde Duiwelsklou wortel is vergelyk met HPLC analises van dieselfde metanolise ekstrakte wat tydens die drogingstudies gebruik is. Twee verskillende NIRS instrumente is gebruik om spektra deur diffuse weerkaatsing in die poeier te meet en kalibrasies is daaropvolgend met gedeeltelike minste vierkante ("PLS") regressie gedoen. Beide onafhanklike data stel validasie en volle kruisvalidasie is gebruik om die prestasie en

voorspellingsvermoë van die onderskeie modelle te evalueer. Uitstekende voginhoud (VI) kalibrasies met 'n standaard voorspellings fout (SVF) van 0.24% (klasinterval: 2.44% tot 10.43%) en 'n korrelasie van 0.99 is gevind. Die model het goed vergelyk met die standaard laboratorium fout (SLF) van 0.14%, oor die gegewe klasinterval.

Met inagneming van die sensitiwiteit van HDVC as verwysingsmetode (SLF = 0.035%), het die HS inhoud kalibrasie (klasinterval: 0.693% tot 2.244%) aanvaarbaar gepresteer met 'n SVF van 0.134%. Die beskikbare klasinterval was egter relatief klein en toekomstige NIRS studies moet gebruik maak van 'n wyer klasinterval indien moontlik. Die 8ρCHG model (klasinterval: 0.069% tot 0.290%) het soortgelyk gepresteer, met 'n standaard kruisvalidasie fout van 0.028% en SLF van 0.007%.

Met die insluiting van monsters van 'n tweede spesie, *H. zeyheri*, is beide die 8ρCHG inhoud en HS:8ρCHG verhouding (klasinterval: 1.84 tot 34.48) kalibrasies as moontlike metodes vir onderskeiding tussen die spesies geëvalueer. Met 'n 8ρCHG inhoud van *ca.* 0.9% het die eerste model 'n goeie skeidingsvermoë getoon. In teenstelling met vorige studies egter, het die verhoudingsmodel minder sukses getoon. Hoofkomponent analise ("PCA") van die spektra was egter baie belowend as 'n kwalitatiewe onderskeidingsmetode.

Volgens hierdie studie kan NIRS 'n waardevolle tegniek wees vir die verdeling van Duiwelsklou in uitvoer klasse op grond van HS inhoud. Hierdie inhoud is egter grotendeels afhanklik van die drogingskondisies en drogingsparameters wat verder verfyn behoort te word om 'n produk van die hoogste kwaliteit met behoud van HS te verseker.

CONTENTS

The language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This dissertation represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

LIST OF ABBREVIATIONS

$\%8\rho CHG_{as\ is}$	concentration of 8ρCHG calculated to moist mass basis, expressed as a percentage
$\%8\rho CHG_{DB}$	concentration of 8ρCHG calculated to dry mass basis, expressed as a percentage
$\%HS_{as\ is}$	concentration of HS calculated to moist mass basis, expressed as a percentage
$\%HS_{DB}$	concentration of HS calculated to dry mass basis, expressed as a percentage
8ρCHG	8-ρ-coumaroyl harpagide
CE	capillary electrophoresis
CLC	column liquid chromatography
CZE	capillary zone electrophoresis
FT-NIRS	Fourier transform near infrared spectroscopy
GC	gas chromatography
HDPE	high-density polyethylene
HPLC	high-performance liquid chromatography
HG	harpagide
HS	harpagoside
ICRA	IdentiCheck™ reflectance accessory
MC	moisture content
MeOH	methanol
MIR	mid-infrared
MLR	multiple linear regression
MS	mass spectrometry
MSC	multiplicative scatter correction
NIR	near infrared
NIRS	near infrared spectroscopy
NMR	nuclear magnetic resonance
PC	principle component
PCA	principle component analysis
PCR	principle component regression
PLSR or PLS	partial least squares regression
PPO	polyphenol oxidase

LIST OF ABBREVIATIONS (continued)

r	correlation coefficient
RH	relative humidity
RMSEP	root mean square error of prediction
S/N	signal to noise ratio
SEC	standard error of calibration
SECV	standard error of cross-validation
SEL	standard error of laboratory
SEP	standard error of prediction
SD	standard deviation
T_{DB}	dry bulb temperature
TLC	thin layer chromatography
T_{WB}	wet bulb temperature
UV	ultra violet

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Chapter 1

Introduction

Chapter 1
Introduction

The genus *Harpagophytum*, of the order Tubiflorae and family Pedaliaceae, containing two species (*H. procumbens* and *H. zeyheri*) and five sub-species (*H. procumbens* spp. *procumbens*; *H. procumbens* spp. *transvaalense*; *H. zeyheri* spp. *zeyheri*; *H. zeyheri* spp. *sublobatum* and *H. zeyheri* spp. *schijffii*), is a prostrate, perennial herb, indigenous to Southern Africa, that grows in semi-arid conditions to an approximate surface diameter of 1.5 m (Watt & Breyer-Brandwijk, 1962; Burger, 1985). The plant is commonly known by various colloquial names including Devil's Claw, Wool Spider and Grapple Plant (Watt & Breyer-Brandwijk, 1962; Smith, 1966).

Although both species have similar chemical compositions, *H. procumbens* has traditionally been preferred and many authors contend that this species exclusively has medicinal value (Baghdikian *et al.*, 1997; Eich *et al.*, 1998; Chrubasik *et al.*, 2000). The medicinal value of this plant is attributed to compounds of the iridoid family (Guillerault *et al.*, 1994; Wu *et al.*, 1998). The main components of interest are two iridoid glucosides: harpagoside (HS) and 8-ρ-coumaroyl harpagide (8ρCHG), that are concentrated in the secondary roots (Guillerault *et al.*, 1994; Baghdikian *et al.*, 1997; George *et al.*, 2001). Typically, HS is present in higher concentrations (*ca.* 2.0%) than 8ρCHG (*ca.* 0.06%) in the root (Baghdikian *et al.*, 1997; Eich *et al.*, 1998). An infusion, prepared from dried, powdered root, is used against a range of ailments, including inflammation, headaches, rheumatism and indigestion (Baghdikian *et al.*, 1997; George *et al.*, 2001). The powdered tubers can either be used directly in a tea infusion or as pills produced by pharmaceutical companies.

One of the problems with the supply of *H. procumbens*, as with many other commercially available crops, is the possibility of adulteration occurring. In this particular case the dried roots of *H. procumbens* may be mixed with that of *H. zeyheri*. Since international trade depends heavily upon the production of consistent quality products, such adulteration could result in serious economic losses during sales and export (Hachfeld & Schippmann, 2000). Currently, approximately 600 tons of dried roots are exported to the United States, Germany and other European countries (Van Wyk & Gericke, 2000; Claassen, 2001). This study will therefore concentrate on *H. procumbens* as source material, with identification based on the quantification of the active compounds that are important for commercial purposes.

A pivotal species-distinguishing factor is the ratio between the iridoids, HS and 8ρCHG. Incorporation of this ratio into rapid analysis techniques would provide efficient detection of the

contamination of *H. procumbens* by *H. zeyheri* roots (Baghdikian *et al.*, 1997; Eich *et al.*, 1998). Besides adulteration, other factors such as ambient conditions and type of processing also seem to affect the concentration and quality of active compounds. This problem has been highlighted by quality control performed by the importing countries, where it was found that some form of degradation occurs that inactivates the HS either during or after export (Baghdikian *et al.*, 1997). It is uncertain whether this loss of activity is due to polymerisation, oxidation or other degrading reactions. However, there may be correlations between the stability of the active principles and the processing conditions applied to the roots (Burger, 1985). Due to the minimal processing of the roots, most of the control should be exercised during the drying phase to avoid losses of the active principles (George *et al.*, 2001).

To establish standard processing procedures, with the aim to guarantee maximum retention of HS in the drug, the influence of different types of processing techniques have to be evaluated. The optimisation of post-harvest treatments and drying procedures of the roots require accurate quantification of the HS content in the final powder. Currently, the preferred method of quantification of HS and 8ρCHG involves the use of high-performance liquid chromatography (HPLC) (Erdös *et al.*, 1978; Sticher & Meier, 1980; Guillerault *et al.*, 1994; Poukens-Renwart *et al.*, 1996; Baghdikian *et al.*, 1997; Feistel & Gaedcke, 2000). This method is, however, costly and time-consuming and can involve the use of high quantities of consumables and dangerous chemicals. Similar problems occur when using other chromatographic techniques such as thin layer chromatography (TLC) or capillary zone electrophoresis (CZE), where special solvent systems may be required to successfully separate the various compounds. It would thus be preferential to develop a rapid analysis method that could be referenced to a standard method such as HPLC or a similarly powerful discriminatory technique.

The use of Fourier transform near infrared spectroscopy (FT-NIRS) technology could provide a possible approach to perform plant analyses without excessive use of chemicals within a very short time. Furthermore, this technique allows for easy and non-destructive measurements (Osborne *et al.*, 1993). HPLC would still be applied as a reference method for calibration to determine the concentrations of the main components of interest, but FT-NIR could eventually replace the chromatographic methods in the case of routine analyses. The FT-NIR prediction model may then be incorporated into the quality control procedures of the dried root and powdered drug. This will allow for the rapid determination of the HS content and may eventually constitute an essential part of the quality control measures used to ensure the quality of the export product. The additional costs needed to implement such quality control measures may be offset by charging higher premiums for such a value-added product that can consistently be guaranteed to conform to international requirements.

The purpose of this study was to determine the effect of various drying conditions on the retention of HS. Three drying methods (freeze, tunnel and sun-drying) and three tunnel-drying temperatures (40°C, 50°C and 60°C at 30% relative humidity) were evaluated. Drying curves were determined that would provide additional information on the behaviour of HS during processing to assist in the future development of an optimal processing procedure for commercial cultivation. Additionally, FT-NIR models that could accurately predict HS content and the ratio of HS:8ρCHG in dried, powdered roots of *H. procumbens* were tested. It has been shown that Devil's Claw has great potential as an export commodity and studies of this nature are important to establish processing guidelines that will ensure the quality of the product.

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Chapter 2

Literature Review

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Literature Review

2.1 Introduction

Popular since ancient times, the use of natural remedies to combat everyday illnesses has re-established itself with fervour in many Westernised cultures. Of late, these cultures have moved towards more homeopathic methods of combating illnesses and other ailments. The use of homeopathic medicines is inherently part of many cultures all over the world, especially among the Oriental cultures such as the Chinese and Japanese (Wu *et al.*, 1998; George *et al.*, 2001). They have developed many herbal remedies using only naturally occurring products and compounds. Indigenous cultures such as the Aboriginals in Australia also maintain a close relationship with their environment and, to survive, they have used whatever nature has to offer them (Anonymous, 2002).

Closer to home, our own San (Bushmen), Khoi (Hottentots) and Bantu cultures developed many ethnic therapies, often utilising unique and indigenous plants (Ragusa *et al.*, 1984; George *et al.*, 2001; Van Wyk, 2002). As technology became more sophisticated, these plant remedies could be characterised, classified and isolated for use in synthetically produced pharmaceuticals (Rajen, 2000). Due to the public interest in such phytopharmaceutical products, many drug companies have a vested interest in these ancient cures and research in this field is ever increasing (George *et al.*, 2001).

One of the most popular internationally recognised plants, with medicinal value, that grows indigenously to Southern Africa, is *Harpagophytum procumbens* DC (Gouws, 1999; George *et al.*, 2001). Two other plant products that have enjoyed great worldwide success are buchu (*Agathosma betulina* Willd.) and various aloe products (*Aloe ferox* L., *A. peglarae* L., *A. littoralis* L.).

The export of these and other phytopharmaceutical products is subject to certain quality parameters and companies that are involved in the trading of these commodities have to provide assurances to their clients. The implementation of laboratory techniques to assay the quality of such products is therefore central to the research and trade in phytochemicals.

2.2 *Harpagophytum procumbens*

Plants of the Genus *Harpagophytum* are commonly known by various names such as Grapple Plant, 'Skerpioendoring' and the German 'Teufelskralle', but mostly as Devil's Claw (Watt & Breyer-Brandwijk, 1962; Smith, 1966). The names are related to the fruit of

the plant that consists of multiple finger-like appendages with a series of curved hooks arranged at random intervals, used for protection and dispersion of the seeds by animals (Figure 2.1). It is a perennial plant (Figure 2.2) that occurs naturally in arid conditions in the Southern part of Africa, in the Kalahari desert and Namibian Steppes (Burger, 1985; Baghdikian *et al.*, 1997). It has been classified in the Order *Tubiflorae*, Family *Pedaliaceae* and Genus *Harpagophytum*, and contains two species and five subspecies (Smith, 1966; Darnley Gibbs, 1974; Burger, 1985). The vernacular name 'Grapple Plant' was first assigned to the species by Burchell in 1811, while the genus was described by A. de Candolle (Smith, 1966).

The medicinal value of the plant was first discovered by the Khoi people. It was applied either externally, as a paste for the treatment of ulcers and other cutaneous lesions, or taken orally as an infusion with purgative value and as an anti-inflammatory agent (Ragusa *et al.*, 1984; Baghdikian *et al.*, 1997; Leung & Foster, 1996). One particular use relates to its analgesic properties, especially for women with menstrual difficulties and for pregnant women who expected a difficult labour (Baghdikian *et al.*, 1997).

The discovery and subsequent introduction of the plant to the Western world is credited to a German farmer, G. A. Mehnert, from the Mariental District of Nababis in Namibia. He obtained samples of the plant after observing its use by traditional Khoi healers in 1904 (Volk, 1964; Ragusa *et al.*, 1984; Mills & Bone, 2000). The subsequent use of the plant by German soldiers provided the basis for its export to Europe where its herbal properties were proclaimed under the name of 'Harpagophytum Tea'. Commercial trade in Devil's Claw commenced in 1953 and thereafter the medical community took interest and investigated the validity of the health claims being made (Volk, 1964; Mills & Bone, 2000).

Commercial cultivation of *H. procumbens* can be achieved by planting seeds or through the use of cuttings that are initially grown in green houses and transplanted to open fields after about 3 weeks (G. Hansford, Grassroots Natural Products, Gouda, South Africa, personal communication, 2001). The plant requires little further attention, but the provision of sufficient water enhances the growth and rate of development. Right from the outset, small secondary tubers are visible on the rhizoidal roots and these may be harvested at any time during the life cycle of the plant. It has, however, been found that the optimum harvesting time occurs after 18 months, when each plant delivers an average of 12 kg of tubers (Figure 2.2). The size of the tubers can vary substantially (Figure 2.3). Both species of *Harpagophytum* are perennial and can therefore survive even after the secondary roots

Figure 2.1 Characteristic dried fruit of *Harpagphytum procumbens* with its multiple finger-like appendages and hooks.

a

b

Figure 2.2 A cultivated *Harpagophytum procumbens* plant showing (a) the flowers and green fruit, and (b) the uncovered secondary roots that are harvested and dried for medicinal use.

Figure 2.3 Secondary roots of *Harpagophytum procumbens* showing the varying sizes of the tubers against a 15 cm scale.

have been removed. For commercial purposes, however, the entire plant is removed once the roots have been harvested (G. Hansford, Grassroots Natural Products, Gouda, South Africa, personal communication, 2001).

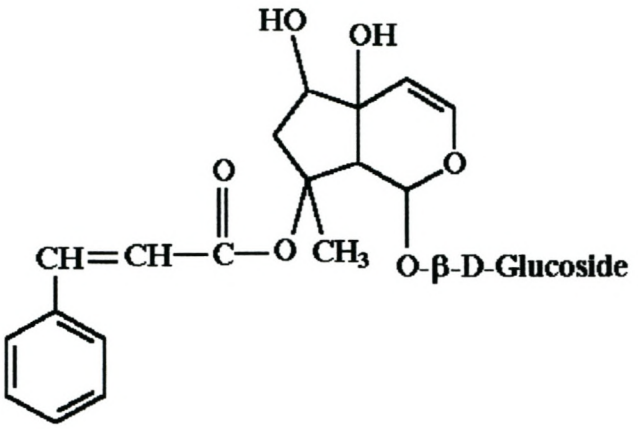
The medicinal value of the roots is apparently concentrated in its four main glucoside iridoids shown in Figure 2.4, namely harpagoside (HS), 8-ρ-coumaroyl harpagide (8ρCHG), harpagide (HG) and procumbide (PC) (Bendall *et al.*, 1979; Burger, 1985; Guillerault *et al.*, 1994; Baghdikian *et al.*, 1999). Of these, HS has the greatest commercial importance and is the most often investigated iridoid constituent of the plant (Poukens-Renwart *et al.*, 1996; Baghdikian *et al.*, 1997; Chrubasik *et al.*, 2000). The study by Lanhers and co-workers (1992) suggested that the pharmaceutical action of Devil's Claw is synergistic and therefore not directly attributable to any specific component.

2.3 Chemical structure and nature of iridoids and other components

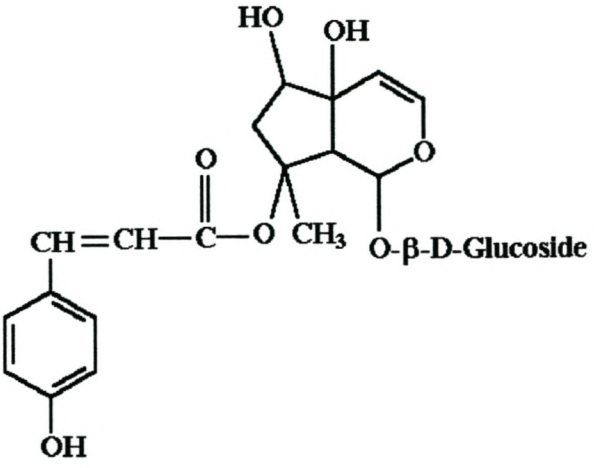
The iridoids as a family generally possess a pyranosyl sugar moiety linked to a non-sugar 5-carbon ring structure (Burger, 1985; Wu *et al.*, 1998). They are termed cyclopentanoids or methylcyclopentanoids and have characteristically bitter tastes and often possess a wide variety of biological activities (Wu *et al.*, 1998). The medicinal value of certain Chinese herbs, such as *Scrophularia ningpoensis*, can be linked directly to their iridoid content (Díaz *et al.*, 1998; Li *et al.*, 1999).

All of the known iridoids have glucose as their sugar moiety and possess a *cis* ring fusion with C1 and C9 protons *trans* to each other (Bendall *et al.*, 1979). Various other configurations were proposed, but using nuclear magnetic resonance (NMR) techniques, with ^{1}H nuclear Overhauser enhancement measurements, the most stable condition was found to be the *cis* ring fusion with protons positioned *trans* to each other. The absolute stereochemistry of the iridoid, harpagide, was determined by Bobbit and Segebarth (1969).

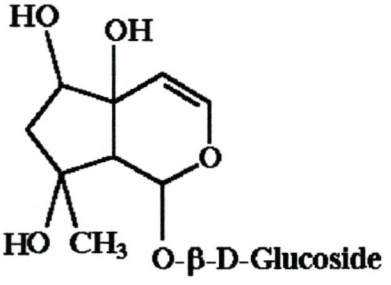
Various researchers have helped to elucidate the chemical composition of *H. procumbens* (Tunmann & Stierstorfer, 1964; Marsh, 1970; Bendall *et al.*, 1979; Burger, 1985). The two components of interest for medicinal and economical reasons are harpagoside (HS), i.e. (1R,5S,6S,9R)-5,6-dihydroxy-1-(6,O,4-hydroxycoumaroyl)-β-D-glucopyranosyl-8-methyl-7:8-oxyrano-3-irideen, and 8-ρ-coumaroyl harpagide (8ρCHG) (Burger *et al.*, 1987; Poukens-Renwart *et al.*, 1996; Baghdikian *et al.*, 1997). Other studies have shown numerous compounds contained within the roots, including the iridoid glucosides procumbide and harpagide, as well as triterpenes (oleanolic acid, ursolic acid), a phenolic acid (cinnamic acid), flavanol glycosides (caempherol, caempherid), a flavone



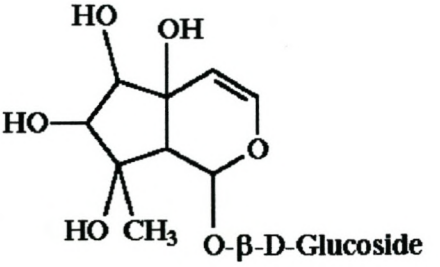
Harpagoside



8-O-ρ-Coumaroyl harpagide



Harpagide



Procumbide

Figure 2.4 Glucoside iridoids of the Genus *Harpagophytum* (Guillerault *et al.*, 1994; Bendall *et al.*, 1979).

(luteolin), sterin-glycosides (β-sitosterin, stigmasterin) (Ragusa *et al.*, 1984; Leung & Foster, 1996), various sugars (glucose, galactose, fructose, raffinose, myo-inositol, saccharose, stachyose) (Ziller & Franz, 1979; Ragusa *et al.*, 1984), phenolic glycosides (acteoside, isoacteoside, bioside), gum resins and trace fatty acids (Ragusa *et al.*, 1984; Burger *et al.*, 1987).

According to Czygan & Krüger (1977) the distribution of HS in the plant is asymmetrical with a high concentration in the roots (and twice as much in the secondary as the primary roots), but only trace amounts are found in the leaves and none in the stems, flowers or fruit (Czygan & Krüger, 1977, Franz *et al.*, 1982). The secondary rhizoidal roots contain HS concentrations varying from 0.5% to 3.0% (Ragusa *et al.*, 1984; Baghdikian *et al.*, 1997; Eich *et al.*, 1998). In comparison to this, studies of commercial root samples from three different distributors in Belgium showed a narrower HS concentration from 1.09% to 1.56% (Poukens-Renwart *et al.*, 1996). From these results, Poukens-Renwart and co-workers (1996), suggested that a suitable minimum level of 1.2% HS be enforced for standardisation of the roots, as stipulated in both the Swiss Pharmacopoeia and Pharmeuropa. Testing of the HS content in both *H. procumbens* (20 samples) and *H. zeyheri* (5 samples), as well as in four commercially available extracts of the drug, showed the HS concentration ranges to be 1.31% to 2.40% (mean value = 1.752%), 0.71% to 1.40% (mean value = 1.090%) and 1.70% to 10.16% (mean value = 4.043%) respectively (Baghdikian *et al.*, 1997). A similar study determined the HS range for *H. procumbens* to be 1.05% to 3.01% (13 samples), while *H. zeyheri* contained 0.70% to 1.56% (10 samples) and commercial drugs and extracts between 1.59% and 3.22% (Eich *et al.*, 1998). Additionally, the 8ρCHG percentages were also determined to be 0.03% to 0.13%, 0.84% to 1.84% and 0.11% to 1.16% for *H. procumbens*, *H. zeyheri* and commercial samples, respectively.

The work of Baghdikian and co-workers (1997), as well as Eich and co-workers (1998), indicates that the harpagoside content does not differ considerably between the two species. Even though there is little to distinguish between the two species on morphological, anatomical or histochemical grounds (G. Hansford, Grassroots Natural Products, Gouda, South Africa, personal communication, 2001), *H. zeyheri* is still deemed inactive with regards to medicinal application. The only major discernable difference is the occurrence of 8ρCHG in *H. zeyheri* in higher levels than in *H. procumbens* (Baghdikian *et al.*, 1997; Eich *et al.*, 1998). If the ratio of HS to 8ρCHG were to be taken, a value of approximately 1:1 would be found for *H. zeyheri*, while *H. procumbens* would be *ca.* 30:1. Some researchers (Czygan & Krüger, 1977; Baghdikian *et al.*, 1997; Baghdikian *et al.*, 1999) do, however,

argue that an infusion of either species delivers similar anti-inflammatory and analgesic effects and that both could therefore be used in the production of commercial drugs.

The ratio between the HS and 8ρCHG concentrations has been successfully used to taxonomically distinguish between the two Harpagophyti species (Baghdikian *et al.*, 1997; Eich *et al.*, 1998). The phytochemical differentiation could therefore be useful for quality control purposes, where the ratio can be used to determine possible adulteration of *H. procumbens* with *H. zeyheri*. Eich and co-workers (1998) suggested a maximum value of 8% 8ρCHG (HS:8ρCHG ratio of 12:1) of the total iridoids below which a sample may be viewed as pure *H. procumbens*. In this case, they viewed 'total iridoids' as the combination between HS and 8ρCHG.

2.4 Medicinal properties

The medicinal properties of Devil's Claw have been the focus of several studies (Erdös *et al.*, 1978; Baghdikian *et al.*, 1997; Baghdikian *et al.*, 1999; Chrubasik *et al.*, 2000; Loew *et al.*, 2001). The anti-inflammatory action of harpagoside was similar to that of cortisone and phenylbutazone, but dose-dependant when compared to indomethacin (Leung & Foster, 1996; Baghdikian *et al.*, 1997). The analgesic effects were comparable to the effect of acetylsalicylic acid (68 $mg.kg^{-1}$ dose). Further studies (Circosta *et al.*, 1984) have also shown reduction of cholesterol and arterial blood pressure, with a decrease in heart rate and protection against arrhythmias. The use of Devil's Claw in these studies has to date shown no short term toxic side effects, although its purgative action may be viewed as being induced through high-dose concentrations, i.e. overdosing (Tunmann & Lux, 1962; Mills & Bone, 2000). Anecdotal evidence suggests the drug to be effective against hyperlipidemia, gastrointestinitis and gout, as well as to possess some sedative activity (Leung & Foster, 1996; Mills & Bone, 2000).

The first studies on the anti-inflammatory properties of Devil's Claw were performed in Germany in 1958 and most of the early work was published early in the 1960's (Tunmann & Lux, 1962; Tunmann & Stierstorfer, 1964; Volk, 1964). Although later studies (Baghdikian, 1997; Eich *et al.*, 1998; Baghdikian *et al.*, 1999; Chrubasik & Eisenberg, 1999) confirmed HS as the compound that provides the medicinal value of Devil's Claw, isolation thereof and its use in purified form showed less or no activity when compared to the natural infusion (Eichler & Koch, 1970; Erdös *et al.*, 1978; Lanhers *et al.*, 1992; Loew *et al.*, 2001). Currently, no clear reason has been found to explain this phenomenon, but recent research (Samal & Geckeler, 2001) has shown that under certain dilution conditions, a diluent may clump together and form large aggregates, rather than dispersing evenly within the solvent.

It could be possible that a minimum aggregation is required before a solution will exhibit biological action and medicinal activity.

The proposed action of the iridoid glucosides in *H. procumbens* is the inhibition of the synthesis of mediators of inflammation (prostaglandins and eicosanoids such as thromboxanes and leucotrines) (Anonymous, 2001). Lower levels of these mediators should reduce both the occurrence of inflammation and the pain associated therewith. One study (Baghdikian *et al.*, 1999) was done to determine the biologically active metabolites of HS, HG and 8ρCHG produced *in vitro* by human intestinal flora. They determined that the human faecal bacteria produced the pyridine monoterpene alkaloid aucubinine B. The anti-inflammatory activity of aucubinine B was not tested. Vanhaelen (1983) has reported that the stomachal digestion of HS or Harpagophyti Tea extract produced harpagogenine as primary active metabolite.

2.5 Analytical systems

Most of the analyses that have, to date, been done on *H. procumbens* and *H. zeyheri*, have used a variety of wet chemistry methods as well as high-performance liquid chromatography (HPLC) (Guillerault *et al.*, 1994; Feistel & Gaedcke, 2000), gas-liquid chromatography (Vanhaelen *et al.*, 1981), high-performance thin layer chromatography (TLC) (Poukens-Renwart *et al.*, 1996) and capillary electrophoresis (Wu *et al.*, 1998). Two of these, TLC and HPLC, as well as near infrared spectroscopy (NIRS) will be discussed below.

2.5.1 *Thin layer chromatography*

Thin layer chromatography (TLC) has developed over the past 40 years into a very effective analytical tool while remaining simple in its methodology. The advances in planar technology in terms of solid phase composition, quantitation, reproducibility and speed of analysis have produced a technique that has been labelled as high-performance TLC (Geiss, 1987). With the advent of newer and more precise analytical systems with higher sample thresholds, such as high-performance liquid chromatography (HPLC), near infrared spectroscopy (NIRS), gas chromatography (GC), mass spectrometry (MS) and various combinations of these, TLC has moved into a more qualitative and supporting role, but it still remains a very important part of analytical chemistry and often forms the basis of many separation studies (Nagels, 1981; Hetem, 1993).

The separation and fingerprinting of a sample by TLC rests on retention times and R_f values in specified solvent systems. Many of the more modern techniques have overcome the problem TLC has with non-specificity (Geiss, 1987). Specificity is the

ability of a technique to selectively isolate a compound or group of compounds, that are of interest, from the rest of the sample "noise" or unimportant information. It can, however, be shown that good laboratory practices and careful use of chromatographic techniques provide very reproducible R_f values that may be correlated to good separation and therefore to identification and discrimination. In addition to the use of R_f values, obtained empirically and cross referenced to literature values, TLC plate results may also be used for purification of impure substances or unknown samples and quantitative work may be performed on the extracted samples (Nagels, 1981; Poukens-Renwart *et al.*, 1996). An advantage of TLC in this regard, is that it can easily be adapted to two-dimensional elution with different solvent systems (Nagels, 1981; Geiss, 1987). This allows for separation of compounds with similar mobility by changing the polarity of the solvents. The most favoured R_f range for quantitative work is from 0.2 to 0.6 and can be selected by the manipulation of these solvents (Wagner & Bladt, 1996). Reproducible R_f values are a prerequisite to quantification (Geiss, 1987).

To date, the most commonly used technique to quantify the constituents of Devil's Claw has been HPLC with UV detection, as evidenced by the numerous publications using this technique (Erdös *et al.*, 1978; Sticher & Meier, 1980; Vanhaelen *et al.*, 1981; Guillerault *et al.*, 1994; Baghdikian *et al.*, 1997; Feistel & Gaedcke, 2000). Recently, especially due to the improvements of TLC methods, some researchers have been using this technique, in conjunction with the usual HPLC, to determine harpagoside content (Poukens-Renwart *et al.*, 1996). The analysis and authentication of Devil's Claw by TLC, and rapid distinction between the species, has also been shown (Czygan *et al.*, 1977; Wagner & Bladt, 1996; G. Hansford, Grassroots Natural Products, Gouda, South Africa, personal communication, 2001). Studies on the iridoid glucoside content of other medicinal plants, such as *Scrophularia ningpoensis*, have also used TLC to good effect (Li *et al.*, 1999).

Liquid chromatography, in general, has an absolute requirement for the trinity of solvent, sorbent and sorbate (Geiss, 1987). Solvent refers to the liquid in the solvent tank, such as methanol or ethyl acetate that, after interaction with the stationary phase, forms a 'pseudo-stationary phase' (mobile phase). Sorbent refers to the stationary phase and in the case of TLC it is the compound immobilised on the plate, such as, alumina or cellulose. Sorbate is the constituent molecules of the sample spotted onto the stationary phase for analysis. The choice of the solvent/sorbent system depends upon the nature and polarity of the analyte.

Silica gel, as a sorbent, is probably the most commonly used stationary phase for TLC analyses (Geiss, 1987; Hetem, 1993). The action of this gel depends upon the occurrence of siloxane groups (–Si–O–Si–), free silanol groups (–Si(OH)–Si(OH)–), reversibly bonded water and free (capillary) water within the gel matrix. The chemical nature of these groups determines the interaction with the sample constituents and thus the chromatographic result. The solvent and sorbate molecules interact with the silanol groups and the degree to which this occurs will determine whether a solute is 'held' by the stationary phase or whether it elutes with the solvent front (Geiss, 1987).

As may be expected, water interaction can have a marked effect on the results of a TLC analysis. Under conditions of high relative humidity (RH), silica plates can show hysteresis for water vapour isotherm adsorption, i.e. different adsorption and desorption isotherm branches (Geiss, 1987). The implication is that if TLC were performed at a high RH, a sorbent will have an R_f value that is distinctly different from a plate run at a lower RH. This creates the problem of incompatible and incomparable results in sequential analyses if the humidity were to be altered drastically. Commercially available pre-coated Merck Silica 60 does not seem to have this problem of hysteresis and can therefore be used without concern. The careful control of relative humidity can, however, also be of use in a TLC system where it can be an excellent activity regulator with hydrophilic sorbents (Geiss, 1987).

The substances separated on TLC plates may be quantified using various methods (Geiss, 1987; Wagner & Bladt, 1996). The plate resolution depends upon the chemical nature of the sample. Visible spots can be measured by either transmittance or diffuse reflectance modes, while spots that are visible only under UV light can only be measured in reflectance mode (Ebel, 1987). An important consideration in this regard is the absorptive nature of silica in the UV spectrum. Some plates may require spraying with a visualising agent such as vanillin-sulphuric acid reagent or anisaldehyde-sulphuric acid reagent (Poukens-Renwart *et al.*, 1996; Wagner & Bladt, 1996). Further possibilities include absorptive analyses of phosphorescence by an inorganic indicator added to the sorbent or by fluorescence of the sample itself. The UV analysis usually concentrates on either 254 nm or 360 nm incidence light values (Wagner & Bladt, 1996).

The actual measurement of the spots for quantitative analysis may be performed by slit scanners or spot scanners, with both single and double beam varieties commercially available, or by densitometry (Ebel, 1987; Poukens-Renwart *et al.*, 1996). For qualitative work or simple screening procedures, analysis by naked eye for either visible or UV light may successfully be employed. These results must be referenced using internal standards

(Geiss, 1987). Experimental results have shown that UV detection has a higher sensitivity (minimum = 0.03 μg harpagoside) than visual (minimum = 0.07 μg harpagoside) (Poukens-Renwart *et al.*, 1996). Further statistical analysis of TLC plates is also possible, especially the use of correlation coefficients and hierarchical clustering of multiple solvent systems under consideration. The results of such a study can be plotted in dendrogram format to reveal optimal solvent combinations for sample identification (Nagels, 1981).

Examples of sprayed (visualised) TLC plates are shown in Figures 2.5, 2.6 and 2.7 (Wagner & Bladt, 1996). In both Figures 2.5 and 2.6 the second channel (2) is the representation of Harpagophyti radix. Figure 2.5 used an ethyl acetate-methanol-water (77:15:8) solvent with vanillin sulphuric acid reagent as spray. Figure 2.6 used an ethyl acetate/glacial acetic acid/formic acid/water (100:11:11:26) solvent system with anisaldehyde-sulphuric acid reagent as visualisation spray. In both cases, clear violet-red zones are formed at $R_f \approx 0.5$ and again at $R_f \approx 0.2$ where they correspond to harpagoside (0.5) and harpagide, procumbide and isoharpagoside (0.2), respectively. The solvent system employed in the development of Figure 2.7 was the same as for Figure 2.5, but the anisaldehyde sulphuric acid spray reagent was used for visualisation. Channel seven (7) represents Harpagophyti radix for Figure 2.7.

Advantages

TLC is a simple, cheap, but very effective method that often requires very little sample preparation before analysis may be done. The system is very flexible in its optimisation through solvent interchangeability and separation medium modification (Geiss, 1987). It provides an easy overview of all sample components (i.e. none are left in a column). This allows for easy screening and thus purity checks. It often has excellent qualitative analysis ability, especially since it lends itself easily to two-dimensional elution (Nagels, 1981). When used correctly, TLC provides a fingerprint of the specific sample being analysed. It is therefore a powerful quality control tool or detection method of adulteration (Geiss, 1987; Wagner & Bladt, 1996). The environmental and chromatographic conditions can easily be optimised to allow for better resolution of only the components of interest. Only a relatively short period of time is required to elucidate most of the characteristic constituents of a complex sample by TLC (Geiss, 1987). Beyond the obvious identification applications, the possibility exists to quantitatively determine some of the constituents, directly from the TLC quantitative analysis (e.g. UV scanning or densitometry), thus allowing for a more detailed and powerful analytical tool (Poukens-Renwart *et al.*, 1996; Wagner & Bladt, 1996).

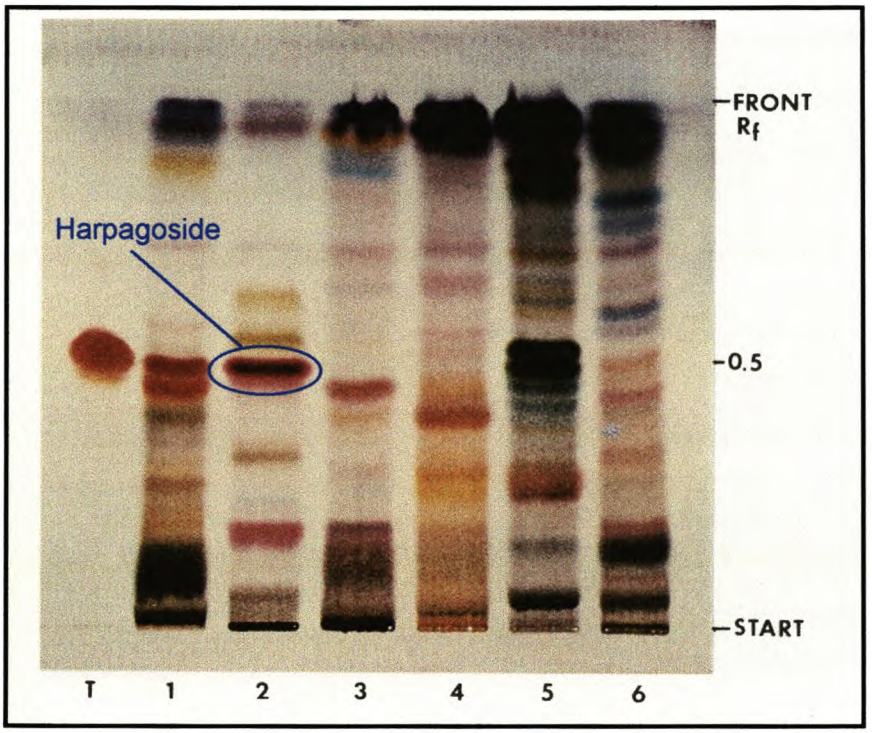


Figure 2.5 Harpagophyti radix sample spotted in channel 2 with Rf ≈ 0.5 representing harpagoside (Wagner & Bladt, 1996). Solvent system was ethyl acetate-methanol-water (77:15:8) and the plate was visualised with vanillin-sulphuric acid reagent.

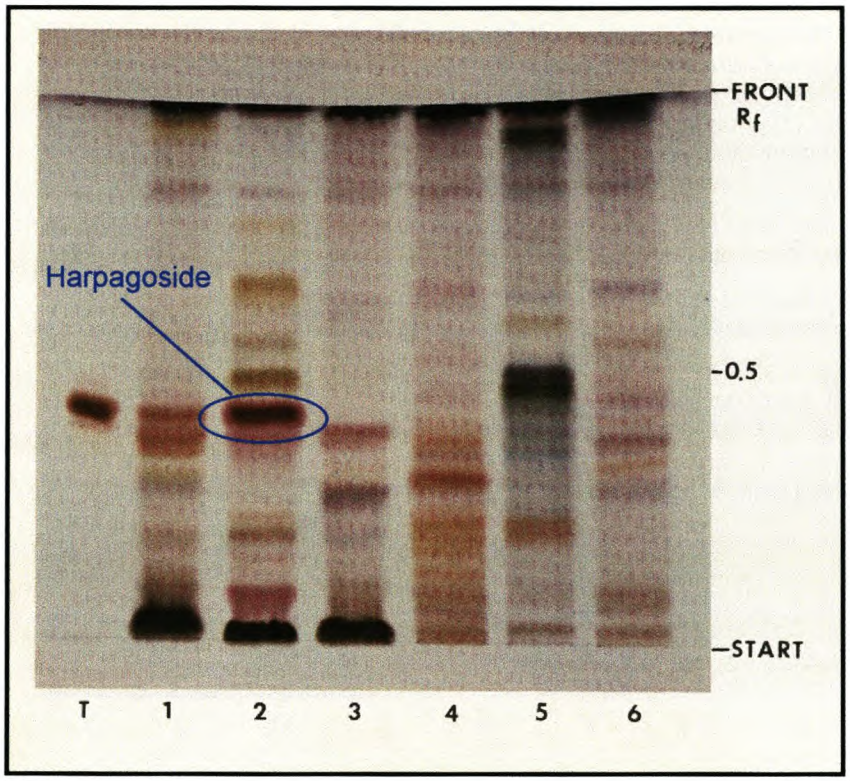


Figure 2.6 Harpagophyti radix sample spotted in channel 2 with Rf ≈ 0.4 representing harpagoside (Wagner & Bladt, 1996). Solvent system was ethyl acetate-glacial acetic acid-formic acid-water (100:11:11:26) and the plate was visualised with anisaldehyde-sulphuric acid reagent.

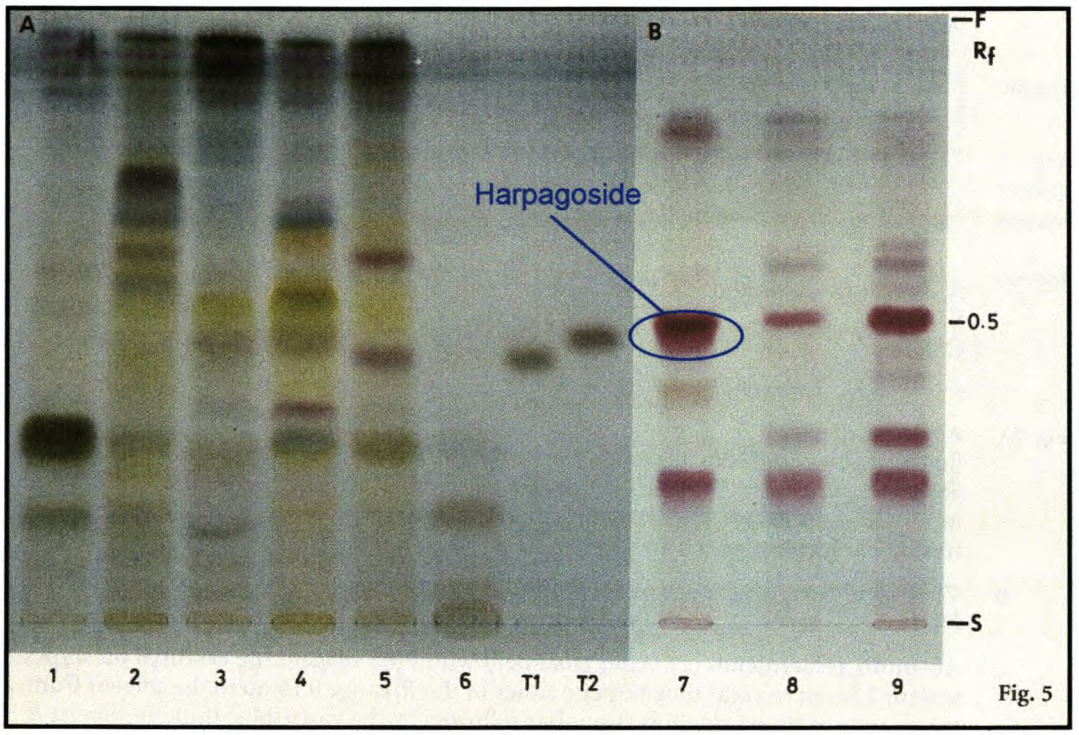


Figure 2.7 Harpagophyti radix sample spotted in channel 7 with Rf ≈ 0.5 representing harpagoside (Wagner & Bladt, 1996). Solvent system was ethyl acetate-methanol-water (77:15:8) and the plate was visualised with anisaldehyde-sulphuric acid reagent.

Disadvantages

TLC, being an open system, is largely affected by environmental conditions (relative humidity and temperature) during analysis and the standardisation of these factors, and therefore the reproducibility of the method, may be difficult (Geiss, 1987). Although it has great qualitative, and in some cases quantitative, ability, TLC results are not always as clear cut as some other chromatographic methods and are thus open to questionable interpretation. Often one is confronted with serious problems of tailing, overlapping zones, band broadening or spreading and imperfect separation. The various eluents and spray reagents used can hold health risks. Chromatography chambers can also be cumbersome to work with. Resolving power is somewhat limited due to the short separation length of the TLC plate. R_f values above 0.75 are not recommended for separation (discriminatory) purposes (Wagner & Bladt, 1996). UV detection may be limited by the properties of the available UV source.

2.5.2 *High-performance liquid chromatography*

High-performance liquid chromatography (HPLC) has over the past 30 years become an integral part of any analytical laboratory. It is a powerful, discriminatory technique that has been utilised for many excellent applications throughout many fields of scientific study (Colin *et al.*, 1986; Tittel & Wagner, 1986; Wu *et al.*, 1998).

The theory behind chromatography has been known and recognised for a long time in scientific circles. Basically it involves the utilisation of interaction principles between the sample and the stationary phase while being exposed to a mobile phase of sorts. HPLC is a refinement of column liquid chromatography (CLC). HPLC has, however, moved away from initial preparative applications (to yield pure substances) to more quantitative and qualitative analyses of samples (Nagels, 1981; Asshauer & Ullner, 1986; Wehrli, 1986).

Most detectors for HPLC are light absorbance detectors (such as spectrophotometers) that are preset to wavelengths or wavelength ranges (Asshauer & Ullner, 1986; Tittel & Wagner, 1986). The specific response of individual detectors cannot be predicted and the use of standards for every desired peak, subsequently, becomes essential. These reference standards are used to set up standard tables or graphs and these, in turn, serve as a means of comparing samples and identifying peaks.. As in most other chromatographic methods, either the peak area or peak height is used for integration and further quantitative or qualitative analysis, but once again these areas need

to be referenced to predefined concentrations of standard reference solutions (Tittel & Wagner, 1986).

The detection of sample concentrations can be temperature dependant and certain literature sources provide data on the effect of temperature variation on various solvents (Berridge, 1985; Asshauer & Ullner, 1986, Sadek, 1996). In modern HPLC systems, however, temperature control over both the detector and column is usually available.

Four types of evaluations can be performed on HPLC data. The first involves the use of an external standard and provides an absolute calibration superimposed on the empirical data by mathematical or graphical means (Asshauer & Ullner, 1986). The second method uses an internal standard that mainly compensates for variations of injection volumes between standards and samples run individually (as in the external method). A third method is called the Area Normalisation Method or 100%-Method. This method involves calculating the sum of all peaks and assuming this result to be 100% of the initial sample. Proportional analysis may then be performed to determine individual contributions by each component. The 100%-Method is, however, more applicable to GC than to HPLC due to detector limitations. Finally the Addition Method produces a linear response to artificially added concentrations of the desired constituent, *h*. This signal response is plotted and regressed to intercept the *y*-axis and provide the concentration of component *h* in the original sample. This method is followed for integration of the smaller peaks within the chromatogram that are often prone to shifting or overlapping (Asshauer & Ullner, 1986).

On the practical side HPLC, as it is used in pharmacology and the pharmaceutical industry, has shown great potential in the development of some products or their formulations (Tittel & Wagner, 1986; Wu *et al.*, 1998; Drøhse Høgedal & Mølgaard, 2000). HPLC has also served as a quality control method for raw materials and the final product and as a method to isolate pharmaceuticals (Díaz *et al.*, 1998). It is important to remember that the work done on such synthetic and near-pure compounds is much simpler than work on plant extracts. Plant extracts form a complex mixture of often unidentified and unknown compounds that can have numerous interactions that complicate the resolution. To facilitate the production of well separated chromatograms, Tittel & Wagner (1986) recommended that extract purification and enrichment form an integral part of sample preparation.

HPLC has been widely used in the analysis of Devil's Claw (*Harpagophytum procumbens* and *H. zeyheri*) and although some of the results have differed considerably, it is still currently viewed as the best quantitative method for the analysis of this product

(Erdös *et al.*, 1978; Sticher & Meier, 1980; Vanhaelen *et al.*, 1981; Guillerault *et al.*, 1994; Poukens-Renwart *et al.*, 1996; Baghdikian *et al.*, 1997; Feistel & Gaedcke, 2000). In most of these cases, the HPLC is coupled to an ultra-violet/visible detector, where mostly the ultra-violet range is used. Additional information may be obtained by using the visible light range of the detector or by using other detection techniques such as mass spectrometry (MS).

For column chromatography, more non-conventional stationary phases such as bonded non-polar C_{18} and C_8-modified silicas have been used in the past (Tittel & Wagner, 1986; Geiss, 1987). It has, however, been suggested that the use of such non-conventional phases, especially with complicated solvent mixtures, are unnecessary and that most separations may be performed using simpler and more cost-effective systems (Geiss, 1987).

Although isocratic elution has been used, the trend for plant extracts and other complicated pharmaceutical mixtures seems to be solvent gradient elution (Tittel & Wagner, 1986; Drøhse Høgedal & Mølgaard, 2000; Feistel & Gaedcke, 2000). The gradient elution allows for finer resolution between peaks of constituents with very similar chemical structures and, therefore, polarity.

Advantages

HPLC or CLC in general has very high resolving power due to the increased length of columns and high theoretical plate counts that can be optimised by various manipulations of the column and solvent conditions (Geiss, 1987). It has excellent quantitative ability and is able to effectively resolve compounds that cannot be separated by GC (Vanhaelen *et al.*, 1981). CLC is less affected by environmental conditions than, for example, thin layer chromatography (TLC) and requires the control of a smaller number of unknown or ill-defined parameters. The use of gradient elution is more easily done than for TLC and is often preferred for the resolution of more complex mixtures. The system lends itself more easily to automation and most modern systems have computerised control available.

Disadvantages

When compared to other chromatographic techniques, HPLC is quite labour and time intensive. It is also a destructive technique and large amounts of various environmentally damaging chemicals are typically used (Geiss, 1987). HPLC has difficulty in broad spectrum application due often to a lack of effective detection means. The separation media and solvent systems are less easily adapted (closed system problem), and thus

HPLC is not always very effective in qualitative applications (Nagels, 1981; Geiss, 1987). Once the method is set, it becomes expensive and time-consuming to change solvent and gradient profile settings to try to increase selectivity and resolution (Geiss, 1987). The columns are very expensive and are often not very robust, i.e. can easily be irreparably damaged. Separations of especially long chain polymers, or mixtures containing such compounds, can become very time consuming.

2.5.3 *Near infrared spectroscopy*

Near infrared spectroscopy (NIRS) relies on the use of near infrared light of wavelength (λ) 800 nm to 2500 nm (wavenumber (ν) of 12 500 cm^{-1} to 4000 cm^{-1}) to obtain an absorbance spectrum (Osborne *et al.*, 1993; Williams & Stevenson, 1990). This spectrum is created after the radiant energy of NIR light passes into (reflectance and transflectance) or through (transmission) a given sample. The chemical and physical structure and composition of the sample matrix ensures that specific, characteristic wavelengths of the light are absorbed. The intensity of these absorptions is related to the subsequent energy states of the sample (Osborne *et al.*, 1993). The molecules of such a sample may begin to vibrate or rotate and, if the energy is sufficient, electrons may even be liberated.

The first application of near infrared spectroscopy in food can be traced to 1938, but thereafter NIRS took a backseat to other analytical methods that could deliver more unequivocal results, especially regarding the elucidation of molecular structures (Williams & Stevenson, 1990). It was only in the early part of the 1960's when NIRS resurfaced and started to find true application in the food industry. During the last decade of the 20th century, great advances in instrumentation and data acquisition gave NIRS a definite place among the other established analytical methods (Wetzel, 1998). As technology and equipment become more refined, companies are utilising NIRS in on-line, as well as in-line applications (Komatsu *et al.*, 1995). More and more diverse fields are using this analytical method for quality control and process management purposes (Wetzel, 1983; Osborne *et al.*, 1993; Wetzel, 1998). Some of the applications, within just the food sector, include the industries of wheat (Ghaedian & Wehling, 1997; Wesley *et al.*, 1999; Cozzolino *et al.*, 2000), wine (Gishen & Dambergs, 1998; Manley *et al.*, 2001), meat processing (Rannou & Downey, 1997; Pink *et al.*, 1999), edible oils (Sato *et al.*, 1998; Che Man & Setiowaty, 1999; Moh *et al.*, 1999; Velasco *et al.*, 1999) and dairy processing (Rodriguez-Otero *et al.*, 1997). It has also found application within the pharmaceutical and drug analysis sectors of the medical community (Ren & Chen, 1999). The application

thereof within drug research, allows for its use in elucidating chemical composition and biochemical reactions and activities.

When compared to the mid-infrared (MIR) region (3000 nm to 20 000 nm; 3334 cm^{-1} to 500 cm^{-1}) an NIR spectrum exhibits an apparent lack of well defined, sharp peaks (Williams & Stevenson, 1990). Mid-infrared spectroscopy is typically used to determine the molecular composition of a sample. If light from the MIR region, and of the correct frequency, is absorbed by the chemical bonds between atoms in a molecule, these bonds begin to vibrate. The molecules have discrete and characteristic bending and stretching vibrations that are associated with their specific configuration and the frequency of the light that they absorbed (Williams & Stevenson, 1990; Osborne *et al.*, 1993). It is possible, by referencing published data, to assign specific peaks in the spectra to specific molecules or bonds and so determine the chemical composition of the sample.

If the molecules in the sample are subjected to bombardment of light from the NIR region, they will experience a great number of weak absorptions that will overlap, effectively creating broad absorption bands in the spectrum (Wetzel, 1983; Williams & Stevenson, 1990). Although electronic transitions are possible for this region, the true value of NIRS lies in the occurrence of overtones and combinations of these and fundamental vibrations that originated in the MIR region (Wetzel, 1983; Wetzel, 1998). Due to their nature, these overtones and combinations are weaker than the electronic transitions (from the ground state to an excited state) that are typical of ultra violet and MIR absorptions (Wetzel, 1998). Besides the lower energy of the incident light, overtones by definition become sequentially weaker as they increase. The overtone pattern of any fundamental vibration may be predicted by dividing the fundamental wavelength by 2, 3, 4, …, n to produce the first, second, third, and n^{th} overtones, respectively (Wetzel, 1983). For this reason, only molecules that absorb MIR light between 5000 nm and 8000 nm will produce overtones that are detectable in the NIR region (Wetzel, 1983; Wetzel, 1998). This implies that the spectra that may typically be generated by NIRS consist of chemically simple molecules that have strong inter-atomic bonds. The strength of these bonds are related to the difference in atomic mass of the atoms, such as for molecules containing carbon (C), nitrogen (N) and oxygen (O) bonded to hydrogen (H), i.e. the most common molecules found in food.

The 'weakness' of the incident light in NIRS analysis produces only small spectral differences between different samples (Wetzel, 1983). These differences may often be too small to be visually apparent and the true value of NIRS as an analytical tool rests on the mathematical treatment of the digital data that produces the spectrum (Wetzel, 1998). In a

Fourier-transform NIRS system, the Fourier-transform mathematics relates the difference in incident light intensity to the reduced intensity after interaction with the sample and a spectrum of peak (or areas of) absorbance is produced. These spectra may be further manipulated by statistical and mathematical methods (collectively, chemometrics) to find correlations between the optical differences and the inherent sample differences.

Instrumentation

When considering the instrumentation, a variety of possible configurations exist. These include discrete filter and grating monochromator instruments, as well as photodiode array and Fourier-transform systems (Osborne *et al.*, 1993; Wetzel, 1998). To produce a Fourier transform near infrared (FT-NIR) spectrum, the most important part of the instrumental design, is the Michelson interferometer that splits the entire source incident ray into two parts with a semi-transparent beam splitter (Figure 2.8) (Van de Voort, 1992; Wetzel, 1998). The two parts are recombined after one beam undergoes variation in its path length. This variation, as a function of time, induces an interference into the recombined beam that will eventually produce an interferogram. After interaction with the sample, certain characteristic wavelengths are absorbed and removed from the interferogram. Once Fourier transformation has taken place, what remains, as data, is the spectrum. The interferogram is subjected to the Fourier transform (an exponential function of the difference between the two mirrors) that produces a 'normal' emittance spectrum. The emittance spectrum can be converted easily to either transmission or absorbance spectra for digital display or plotting.

Type of application

Although the initial applications of NIRS were limited to qualitative measurements of pure chemicals and simple mixtures, the advent of chemometrics provided the means to quantify samples and diversify the type of measurements to include granular or powdered samples (Wetzel, 1983; Wetzel, 1998). The quantitation of such granular samples is achieved by diffuse reflectance and may successfully be employed if the analyte is present at a level of 1% or more and if multivariate statistical approaches are used for data collected at multiple wavelengths (Wetzel, 1998). If a simple Beer's Law equation is used to describe the contributing factors of a spectrum, absorbance at any wavelength will be a function of the path length that the light travels and the concentration of the analyte being

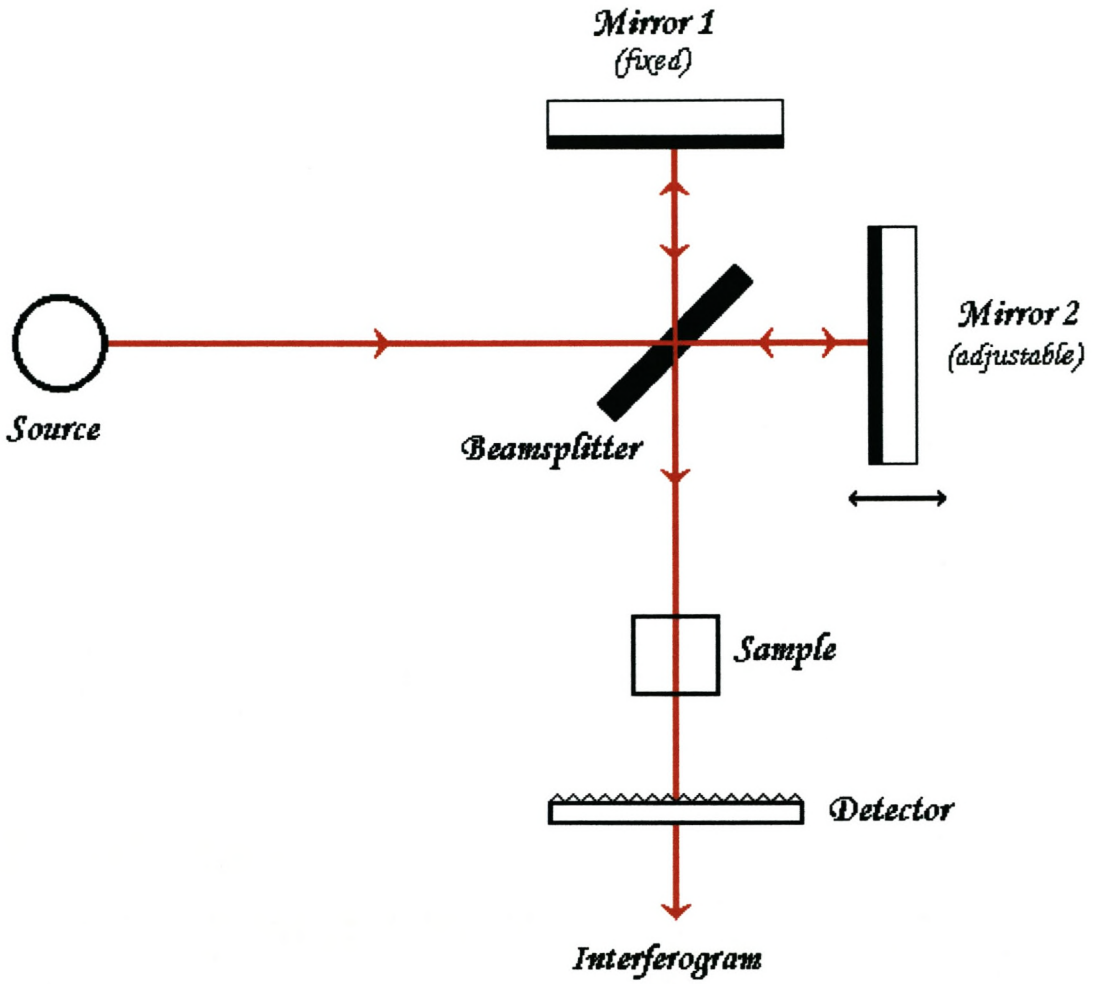


Figure 2.8 Schematic representation of the action of a Michelson interferometer in a Fourier-transform near infrared spectrophotometer (Van der Voort, 1992).

measured (Wetzel, 1998). For transmittance measurements of liquids, the path length is a constant determined by the thickness of the sample cuvette, but diffuse reflectance has to contend with changes in both path length, due to scattering, and concentration.

The scattering of light, as it passes into a granular sample, is caused by the occurrence of a variety of surfaces that are placed at random angles to each other, from which the light bounces. When light that has interacted with a sample is scattered and redirected back along the path of incidence to the detector, diffuse reflectance occurs. Scattering enhances the intensity of light returning to the detector, but also increases the variability of the baseline due to the multipath effect (Wetzel, 1998). This effect describes the detection, by diffuse reflectance, of light that is a combination of both absorbed (interaction with the sample) and scattered light (no interaction with the sample) (Wetzel, 1983). This can have a large influence on the generated spectrum, since the ratio between reflected light (absorbed and scattered) and incident light determines the absorption profile. Sample presentation is therefore extremely important to minimise the scattering effect (measured as false absorbance) and, as far as possible, to keep the level of scattering constant for each sample (Wetzel, 1983; Williams & Stevenson, 1990).

The influence of the particle size of granular samples on acquired spectra may be illustrated by Figure 2.9 (Wetzel, 1983; Osborne *et al.*, 1993). The observed effect may be attributed to Rayleigh (elastic) scattering (Wetzel, 1983). The coarser the sample matrix, the lower the reflectance and greater the difference between the spectrum and the baseline. Although this effect may be of great practical value to discriminate between coarse and fine samples, it may be compensated for by baseline corrections that also increase the relative size of small peaks and enlarge minor differences in large peaks (Wetzel, 1983; Wetzel, 1998).

Calibration development

Near infrared spectroscopy initially involves calibration of the instrument through the use of a sample and validation set (Wetzel, 1983; Osborne *et al.*, 1993). The development of a prediction model is achieved by reducing the spectral data of the sample set to a 'best-fit' equation (Wetzel, 1983). This equation uses the best possible set of coefficients to predict the concentration of the analyte at each wavelength. It is calculated using certain global linear regression techniques, such as principle component regression (PCR), partial least squares regression (PLSR) and multiple linear regression (MLR) (Wetzel, 1983; Workman, 1992; Osborne *et al.*, 1993; Wetzel, 1998).

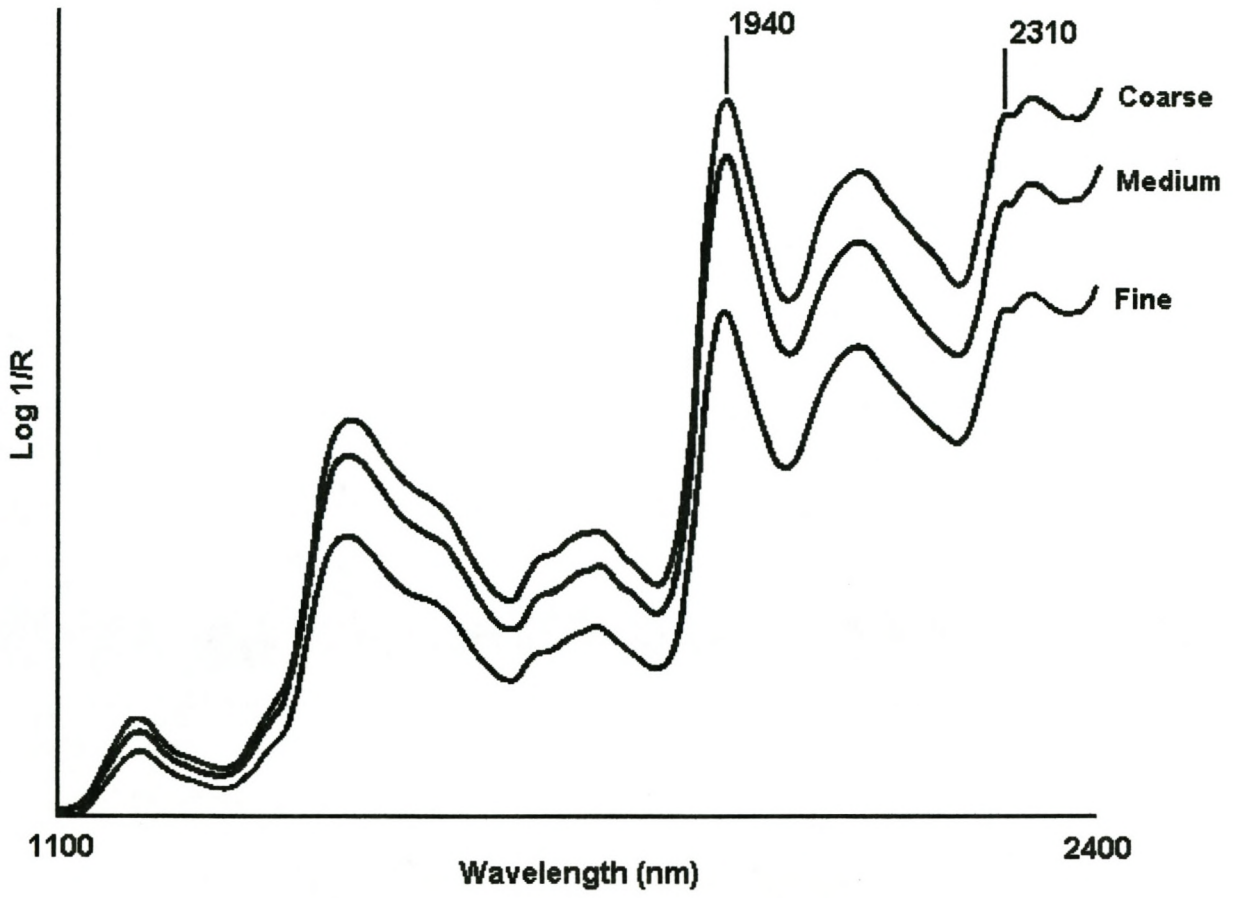


Figure 2.9 Influence of particle size variation of ground wheat on near infrared spectroscopic analysis (Osborne *et al.*, 1994).

The accuracy of a calibration depends, to a great extent, on the accuracy of the reference values upon which it is built (Wetzel, 1983). These reference results are usually determined by wet chemistry (proximate) analysis, high-performance liquid chromatography (HPLC), gas chromatography (GC) or capillary electrophoresis (CE), against which the spectral data is regressed (Wetzel, 1998). The standard error of laboratory (SEL), determined by the difference between blind duplicates (Equation 2.1), measured by the reference method, is used as a bench-mark for the performance of a new calibration (Workman, 1992; Wetzel, 1998).

$$\text{SEL} = \sqrt{\frac{\sum_{1}^{n}(y_1 - y_2)^2}{2n}} \qquad \ldots 2.1$$

where y_1 = first analysis

y_2 = second (duplicate) analysis

n = total number of samples (not analyses)

Once the calibration and regression have been performed, the standard error of calibration (SEC) or standard error of estimate (SEE) can be determined by the difference between a predicted value and the true, reference value for samples within the calibration set (Equation 2.2) (Workman, 1992).

$$\text{SEC} = \sqrt{\frac{\sum_{i=1}^{N}(y_i - \hat{y}_i)^2}{N - K - 1}} \qquad \ldots 2.2$$

where y_i = reference value

$\hat{y}_i$ = predicted value

K = number of wavelengths used

The performance of a calibration improves if the SEC approaches the SEL. To achieve this shift in the SEC, pre-processing of the spectral data may be done by applying a variety of algorithms. A new calibration, using the pre-processed data, is built and a new SEC is calculated and compared to the SEL. A further statistical performance indicator, the standard error of prediction (SEP; Equation 2.3), is calculated when the analyte values of the independent validation set of samples are predicted by the regression equation formed from the initial calibration (Wetzel, 1998). This implies that the same equation (2.2) as for

SEC is used, but that the predicted values now fall outside of the calibration set, i.e. in the validation set (Workman, 1992).

$$\text{SEP} = \sqrt{\frac{\sum_{i=1}^{N}(y_i - \hat{y}_i)^2}{N - K - 1}} \qquad \ldots 2.3$$

The F-value of the regression, an indicator of the signal to noise (S/N) ratio, should also be maximised and may be used as a good indicator of the success of the regression equation in predicting analyte values (Wetzel, 1998).

Data pre-processing

Maximising the significance of any spectral differences is the main aim of using the pre-processing algorithms (Osborne *et al.*, 1993; Hopkins, 2001). Achieving this becomes very difficult if the signal to noise (S/N) ratio of a given spectrum is very small. As NIRS utilises light of lower intensity it will necessarily produce less sensitive responses in the excited molecules (Wetzel, 1998). To compensate for this lower sensitivity, the noise levels detected by the instrument have to be kept to a minimum to create a high S/N ratio. The noise levels are influenced by the instrumentation, sample preparation and presentation and by the environmental conditions within which the spectra are generated (Wetzel, 1983). Environmental conditions are especially important, as the effect that they will have on the weak absorbing bands of NIRS will be much more pronounced than for those regions that produce strong absorptions.

One of the most common pre-processing algorithms used calculates the first and second derivatives of each spectrum (Wetzel, 1998). Derivatives are used to enhance slight spectral differences and thus allow for better recognition of different samples by the regression equation. The use of such derivatives can also compensate for baseline shifts that have been caused by light scattering. Further pre-treatments include Savitsky-Golay or other smoothing algorithms that reduce the effect of noise on the spectrum, subtraction of a background spectrum to remove common environmental noise and baseline corrections that aid the visual comparison of spectra.

Normalisation is another method of removing bias from a spectrum without necessarily using derivatives (Wetzel, 1998). This may be achieved by mean centring the spectra (subtracting the average absorbance from each spectrum) and then establishing unit variances by dividing the absorbance by the standard deviation. A second

normalisation procedure is multiplicative scatter correction (MSC). This approach also uses mean centring, but follows this by curve fitting the spectrum to the average and dividing it by the curve fit value. MSC is very useful to normalise spectra from samples that contain particles of inconsistent size (e.g. powders).

Only one study could be found that specifically addresses the use of NIR technology in relation to *Harpagophytum procumbens* and its constituents (Schulz *et al.*, 2002). This was, however, only a feasibility study based on ethanolic extracts of Devil's Claw root. Other recent papers describe similar research concerning studies on phytopharmaceuticals, as well as natural or herbal plant remedies, and NIR (Ren & Chen, 1999; Schulz *et al.*, 1999; Huck, 2002). One of the research groups used a dispersive Foss NIRSystems 5000 in the measurement of certain phenolic and alkaloid substances (HPLC reference method) in green tea leaves (Schulz *et al.*, 1999). A second study concentrated on the quantification of ginsenosides in American ginseng (Ren & Chen, 1999). The detection of eleven individual ginsenosides, as well as total ginsenosides was evaluated. The last study evaluated NIRS for the quantification of hypericin in *Hypericum perforatum* (St. John's Wort) extracts (Huck, 2002).

Advantages

NIRS has several advantages over other analytical systems. It is a rapid, easily used technology that is non-destructive in its nature (Osborne *et al.*, 1993; Wetzel, 1998). It does not require chemical consumables, is environmentally friendly, very fast and produces a complete spectrum of constituents in the sample. The 'weakness' that is typical of NIR absorptions simplifies the spectra and restricts the information extracted to fundamentally strong chemical bonds between light atoms. This implies that NIRS would typically be describing the CH, NH and OH bonds that are the fundamental building blocks of organic molecules (Wetzel, 1998). Repetitions of absorbing species (i.e. further overtones or combinations) in the spectrum allows for elucidation of spectral variation in the cases where the initial bands may be obscured by strongly absorbing species such as water. Chemometric manipulations of the data can be numerous and very revealing, if handled correctly. It is possible to use glass for windows, sample cells and lenses. This is cheaper and easier than the alkali salts (such as KBr and KCl) typical of MIR analyses. It is also possible to create a pseudo-homogenous spectrum from an non-homogenous sample by scanning a large area of the sample and averaging the sample's properties. The relatively deep penetration of NIR light into samples also affects better representation of

the chemical properties. Once the method has been developed, the instrument can easily be used by technicians for process control purposes.

Disadvantages

Near infrared spectroscopy requires an extensive calibration and validation sample set to produce robust prediction models. The reference method often requires a lot of time for sample collection, preparation and presentation and may involve large expenses, depending upon the nature of the analyte. The spectra that are generated in the NIR region are complicated, consisting of a great many combinations of fundamental vibrations and overtones (Wetzel, 1998). The overlapping of these bands makes qualitative analysis directly from the spectra very difficult. If quantitative analyses are required, a detailed knowledge of the mathematical models and algorithms used, is required. Although, in principle, diffuse reflectance uses light scattering to its advantage, the effect has a greater influence on the spectrum for the NIR region than for the MIR region, and careful signal to noise control is required. For this reason, instruments are less easily adaptable to extreme processing conditions that are typical of factory environments and thus can make the commercial implementation of NIR technology problematic. Effective use and development of new calibrations requires sufficient training of the operator. Furthermore, the sustainable maintenance of a calibration set is very difficult and this lack of sample stability over long periods of time causes problems for calibration transferring.

2.6 Conclusions

Although certain researchers have disputed the purported medicinal value and activity of Devil's Claw, one of its constituents, the gluco-iridoid harpagoside, has been implicated in the potency of this product. With current annual exports between 500 and 600 tons of dried *Harpagophytum procumbens* root, the market for this product is already recognised and commercial cultivation of *H. procumbens* has been established in Namibia and other parts of Southern Africa. Plant improvement studies are also done at Gouda under the auspices of Grassroots Natural Products.

Advances in modern analytical techniques have provided excellent means to perform studies of qualitative and quantitative nature. Various combinations of sensitive techniques can be used to provide reliable data covering the entire scope of constituents in a sample. In the case of studies on the harpagoside content of *Harpagophytum procumbens*, HPLC and CE studies, along with data from NMR studies, have been very successful. A rapid and non-destructive technique, near infrared spectroscopy, has shown great potential for the analysis

of food related products, and in some cases has successfully replaced established analytical methods such as HPLC for routine analyses.

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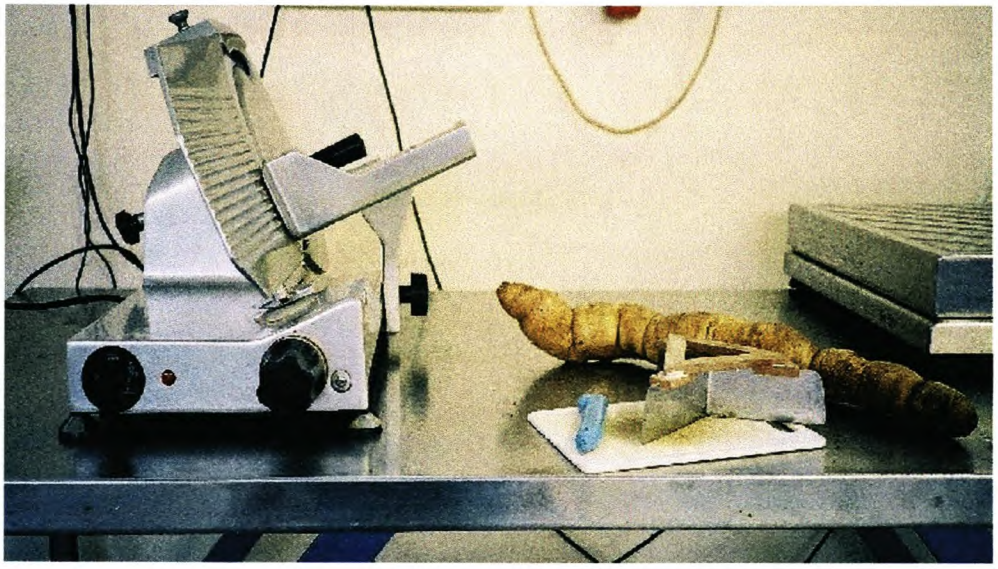
Chapter 3

Evaluation of the effect of drying method and drying temperature on the harpagoside content in dried *Harpagophytum procumbens* root



Chapter 3

Evaluation of the effect of drying method and drying temperature on the harpagoside content in dried *Harpagophytum procumbens* root

Abstract

The retention of harpagoside (HS), a glucoside iridoid found in Devil's Claw (*Harpagophytum procumbens*) root, is of importance both to producers and consumers of the product as it has been implicated in the purported medicinal activity of the product. Although only limited processing of the root occurs, drying or dehydration forms an integral part of the process. This may lead to degradation of HS and, for the first time, the effect of specific drying methods and conditions on the retention of this compound was investigated. Current drying practices often involve uncontrolled sun-drying of Devil's Claw root. High-performance liquid chromatography was used to quantify HS and a 91.70% recovery was shown. This study has indicated that sun-drying of samples (8 × 3 methods) differed significantly ($P < 0.05$) from either tunnel-drying (60°C, 30% relative humidity (RH)) or freeze-drying. Although the freeze and tunnel-drying results did not differ significantly ($P > 0.05$), the best retention of HS was achieved by freeze-drying. Due to the high costs involved in freeze-drying, tunnel-drying was further investigated as a commercially viable alternative and the effects of various temperatures (40°C, 50°C and 60°C) at a constant 30% RH were evaluated. Significant differences were found between samples (9 × 3 temperatures) dried at 40°C and 50°C, with the best retention of HS at 50°C. Drying at 60°C did not differ significantly from the other treatments, but the retention of HS was less than at 50°C. Further investigation of the mechanisms of HS degradation is needed before implementation in practice, but the study demonstrated that optimisation of the drying conditions for Devil's Claw root is required to prevent unnecessary HS losses.

Introduction

Drying of either natural or processed products could have an effect on the composition of the product. This effect can either be desirable or undesirable. Depending on the drying conditions, case hardening, discolouration, enzymatic reactions, oxidation and/or polymerisation may occur (Thijssen, 1979; Hallström & Skjöldebrand, 1983). These effects may or may not have an influence on the sample characteristics, but when dealing with herbal products the retention of the medicinally valuable components should be prioritised. Harsh drying conditions, i.e. large temperature fluctuations or high drying temperatures, will affect the retention of such components (George *et al.*, 2001). In the case of Devil's Claw (*Harpagophytum procumbens*), drying is

essential for the preservation of the root. The final moisture content of the exported dried root is not regulated, but a moisture content of 10% or less is recommended to prevent microbial spoilage. Very little information is, however, available on the stability of the active compound, harpagoside (HS). It is possible that degradation during drying and storage could lead to the loss of HS along with observed discolouration.

Natural or sun-drying is probably the most difficult drying technique to control. Even though it may be preferred for the obvious economic benefits, it is highly dependant on climatic conditions. Other techniques, such as tunnel-drying or freeze-drying, are much more controlled and therefore less prone to irregular drying. They are preferred to ensure the production of a more constant quality dried product (Von Loesecke, 1945; Lorentzen, 1983).

The flow of air over the product, in conjunction with the actual temperature it is exposed to, can greatly influence the physico-chemical changes that occur (Chirife & Cachero, 1970). With tunnel-drying, parameters such as air flow, temperature and relative humidity (RH) must be properly controlled to ensure a quality product, especially if thermo-labile constituents are present. Freeze-drying is especially recommended when considering thermo-labile constituents, since the product is subjected to low drying temperatures (George *et al.*, 2001).

Currently, the drying of Devil's Claw consists of either sun-drying or tunnel-drying at *ca.* 60°C and 30% RH (G. Hansford, Grassroots Natural Products, Gouda, South Africa, personal communication, 2001). Drying is terminated when a brittle product (usually disk-shaped slices of root) is obtained.

High-performance liquid chromatography (HPLC) analyses have mostly been used to determine the HS content of the root (Guillerault *et al.*, 1994; Feistel & Gaedcke, 2000). Since the medicinal properties of the root are linked to the HS content (Ragusa *et al.*, 1984; Leung & Foster, 1996), the measure of this compound would provide a platform whereby comparisons could be made. A variety of extraction methods for HPLC analysis of Devil's Claw are mentioned in the literature. Lanhers *et al.* (1992) used only aqueous extracts, while others used either a combination of methanol (MeOH) and water extraction (Guillerault *et al.*, 1994) or just MeOH extraction (Baghdikian *et al.*, 1997).

This study evaluated the relative effect that three drying methods (sun, tunnel and freeze-drying) had on the harpagoside (HS) content of Devil's Claw. Due to a general preference for the tunnel-drying method, a further comparative study was included that evaluated the effect of drying temperature (40°C, 50°C and 60°C) on the HS content. Comparisons were made based on the HS content as determined by HPLC.

Materials and Methods

Preparation of samples

Fresh *H. procumbens* secondary roots from cultivated plants were obtained in three batches from Grassroots Natural Products (Plant Improvement Centre, Gouda, South Africa). The first batch (March 2001) contained *ca.* 6 kg of fresh roots and the subsequent batches (July 2001 and November 2001) *ca.* 17 kg to 25 kg each. The irregularly shaped roots varied from *ca.* 25 mm to 120 mm in diameter and each weighed *ca.* 0.07 kg to 1.50 kg. Individual samples did not necessarily correspond to individual roots and smaller roots were often combined to form one sample.

All fresh roots were kept under ambient conditions until processing. Before slicing, the roots were rinsed with tap water to remove any excess dirt. The roots were sliced transversely into 6 mm thick disks (Rheninghaus polony cutter, Italy). These disks were then cut into three triangular sectors of approximately the same size. The three sectors were randomly divided between the three drying treatments to minimise influences from any inter and intra-root HS variation. For comparison of the drying methods *ca.* 450 g of fresh root was dried per sample, with 8 replicates performed.

Effect of drying method

For sun-drying the samples were spread onto wooden drying trays (890 mm × 600 mm) covered with a high-density polyethylene (HDPE) net (2 mm mesh, Netlon™, Tensar International, UK), directly after slicing. A second net was placed on top of the samples and weighed down with bricks to avoid dried samples being blown away. These trays were placed daily on a tarred drying court from 08:00 to 16:30 and then removed to a shed where a fan provided additional air movement during the night. Samples were considered dry when brittle (G. Hansford, Grassroots Natural Products, Gouda, South Africa, personal communication, 2001). Weather data was obtained from the automatic weather station at the Nietvoorbij experimental farm (ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa).

The conditions for tunnel-drying were set at a dry bulb temperature (T_{DB}) of 60.0°C and relative humidity (RH) of 30%, i.e. a wet bulb temperature (T_{WB}) of 39.72°C. A purpose-built experimental dehydrator was used for drying at a maximum airspeed of 3 $m.s^{-1}$. The system was computer-controlled with isothermal temperature control of both the dry and wet bulb temperatures by CAL 3200 Autotune temperature modules (Ana-Digi Systems, Bellville, South Africa). The drying time was fixed at 24 h.

Samples that were freeze-dried were initially frozen at -20°C in plastic trays (170 mm × 115 mm × 30 mm). The frozen samples were then dried for 5 days in an Atlas pilot-scale freeze-drier (Denmark model, Copenhagen, Denmark) at a shelf temperature of 40°C.

Effect of drying temperature

For comparison of the effect of drying temperature on the retention of HS during tunnel-drying, the following conditions at 30% RH were used: T_{DB} of 40.0°C (T_{WB} of 26.09°C), T_{DB} of 50.0°C (T_{WB} of 32.32°C) and T_{DB} of 60°C (T_{WB} of 39.72°C). After slicing, the samples (*ca.* 450 g each) were immediately spread onto stainless steel mesh drying trays (870 mm × 600 mm) covered with an HDPE net, and dried. Three purpose-built experimental dehydrators, one controlled by the CAL 3200 Autotune temperature controller (Ana-Digi Systems, Bellville, South Africa) and the other two configured as described by Hansmann & Van Noort (1992), were used. The last two driers were set to control to a maximum airspeed of 4 $m.s^{-1}$.

Nine replicates were performed per drying temperature, with the replicates balanced between the three drying-tunnels to avoid inter-tunnel variations. A total of 27 individual samples (9 × 3 temperatures) were therefore prepared. To ensure that all samples were dried to approximately the same moisture content, different drying times for each temperature were used.

Termination of tunnel-drying was estimated from drying curves calculated after drying in a small-scale experimental dehydrator. Fresh Devil's Claw root (*ca.* 1.3 kg per sample) was spread out on 50-mesh stainless steel trays (370 mm × 310 mm) and dried at 40°C, 50°C or 60°C, respectively. The trays were mounted in a cage, suspended from 2 single-point Tedea-Huntleigh 1040 load cells (Loadtech Ltd., Centurion, South Africa), the output of which was digitised using an Advantech Adam 4011 analogue input module (ProMicro cc., Bryanston, South Africa). Computerised data acquisition was achieved via an Adam 4520 RS-232/485 converter (ProMicro cc., Bryanston, South Africa) with changes in mass recorded at 2 minute intervals.

Drying rate curves were generated from MS-Excel 2000 spreadsheets and approximated by polynomial least squares fitting using the Curve3 program (programmed by C.F. Hansmann, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa). The drying times were estimated from a plot of moisture content versus time. Once drying in larger tunnels with roots harvested at a later stage commenced, these times proved too short and were subsequently adjusted to 17 h for T_{DB} = 40°C, 11 h for T_{DB} = 50°C and 6 h for T_{DB} = 60°C.

Grinding

To avoid any moisture uptake after drying and before grinding, the samples were vacuum sealed in aluminium laminated pouches (Geiger & Klotzbücher Multivac, South Africa). Grinding of the samples was accomplished using a Retsch rotary mill fitted with a 1 mm sieve size. Just before grinding, the vacuum seals were broken and the dried roots crushed by hand to avoid blockage of the mill funnel. After grinding each sample, the powder was temporarily spooned into a 200 ml plastic screw cap bottle. The mill was brushed and vacuumed, or wiped with a cloth, to remove excess powder before grinding the next sample. Samples were finally stored in 30 ml plastic screw cap vials and sealed in large plastic containers with anhydrous $CaCl_2$ as desiccant.

Extraction

A crude extract was made by weighing *ca.* 0.15 g of dried, ground sample into 20 ml Pyrex glass culture tubes with teflon-lined screw caps (Corning Inc., USA). To each tube, 15 ml of a 70% methanol-water (MeOH-H_2O) mixture was added. The tubes were then shaken and placed in a water bath at 40°C. They were kept in the water bath for 60 minutes, manually shaken at 15 minute intervals and were subsequently cooled to ambient temperature in tap water for 10 to 15 minutes. Samples were decanted into 15 ml graduated polypropylene centrifuge tubes (Merck, South Africa) and centrifuged at 4000 r/min (1788.8 × g) for 12 minutes in a Universal 16 centrifuge (Hettich Zentrifugen, Germany). The supernatant was decanted into 24 ml EPA glass vials with teflon-lined screw caps (Separations, South Africa) and stored at *ca.* 4°C until analysed.

A multiple extraction test was performed on 12 samples by re-suspending the pellet in 14 ml of the MeOH-H_2O mixture and allowing a further 60 minutes for extraction at 40°C. This second extract was centrifuged and the supernatant stored as described earlier. The relative extraction potential of two mixtures (100% MeOH and 70% MeOH-H_2O) was also tested. Here, a duplicate set of 12 samples was extracted, each with one of the mixtures, and the extracts centrifuged and stored as described earlier.

Moisture determination

Moisture content (MC) was determined gravimetrically in duplicate for all samples. All masses were determined to 4 decimals using a Precisa 262SMA-FR electronic balance (Instrulab, Johannesburg, South Africa). Depending upon the amount available for analysis, 3 g to 8 g (mean ± SD: 5.379 g ± 0.616 g) of each dried, ground sample was weighed into nickel moisture pans before drying under vacuum (*ca.* 90 kPa) for 16 hours at 70°C. The moisture content of each sample was calculated as follows (Equation 3.1):

$$\% \text{ Moisture Content} = \frac{(m_b - m_a) \times 100}{(m_b - m_e)} \quad \ldots 3.1$$

where: m_b is the mass of the moisture pan with lid and moist sample (g)

m_a is the mass of the moisture pan with lid and dry sample (g)

m_e is the mass of the moisture pan with lid, empty (g)

High-performance liquid chromatography analysis

HPLC separations were carried out, in duplicate, using a Waters™ LC Module 1 plus system, equipped with a single wavelength UV-visible detector and controlled by Millennium™ workstation software (version 2.15, Waters Corporation, UK). The method was based on the HPLC analysis performed by Feistel & Gaedcke (2000). A Phenomenex® Prodigy 5 ODS-2 column (150 mm × 4.60 mm, 5 µm particle size; Separations, South Africa) was used with a solvent gradient containing only water and methanol (Table 3.1). The methanol (HPLC grade; Chromasolv®, Merck) was filtered using a borosilicate all-glass filter holder with 0.45 µm HV-type filters (Millipore, Ireland). The MeOH-H_2O mixture (10 ml MeOH + 850 ml H_2O) was filtered similarly, using a 0.45 µm HA-type filter. The water was first treated with a Modulab water purification system (Separations, South Africa) where a 5 µm filter removed coarse particles followed by a carbon filter, a reverse osmosis process, a mixed bed deioniser and 0.22 µm filter. As an additional step, this water was also treated with a Milli-Q Académic ultrapure water system (Millipore, Ireland). All solvent lines passed through a Jour Research X-ACT degasser (Separations, South Africa) before entering the system.

The column, with a Jour-Guard RP/C18-5µ guard column (Separations, South Africa), was conditioned and run at 40°C with a flow rate of 0.7 $ml.min^{-1}$. An aliquot of the extract supernatant was filtered through a 0.45 µm HV-type filter (13 mm) before injection. Injection (10µl) occurred automatically from a 48 sample carousel, loaded with 4 ml glass vials (Anatech, South Africa) containing 0.25 ml conical inserts (Supelco, USA). Absorbance was measured at 278 nm (Sticher & Meier, 1980; Guillerault *et al.*, 1994; Baghdikian *et al.*, 1997; Feistel & Gaedcke, 2000).

Standard solutions

The quantification of the HS content of Devil's Claw required a standard curve within the

Table 3.1 HPLC solvent gradient program (adapted from Feistel & Gaedcke, 2000).

Time (min)	Flow Rate ($ml.min^{-1}$)	% Solvent A (100% MeOH)	% Solvent B (MeOH + H_2O; 10 ml + 850 ml)
0	0.70	0	100
6	0.70	0	100
25	0.70	45	55
35	0.70	65	35
50	0.70	100	0
60	0.70	0	100
70	0.00	0	100

expected concentration range of *ca.* 1.0% to 3.0% (Baghdikian *et al.*, 1997; Eich *et al.*, 1998). A dilution series of pure HS (Extrasynthese, Genay, France) for the development of this curve, was prepared with HPLC grade methanol (Chromasolv®, Merck) in the amounts given in Table 3.2. These solutions were injected, in duplicate (10 µl each), as external standards to develop the curve given in Figure 3.1. The following linear regression equation was used to calculate the HS concentrations in the Devil's Claw samples (Equation 3.2):

$$y = 3.919 \times 10^6 x + 61296 \qquad \ldots 3.2$$

where: y is the integrated HPLC area measured in µV.s

x is the analyte concentration measured in µg.10 μl^{-1}

Using Equation 3.2, the analyte concentration, in µg.10 μl^{-1}, was calculated. To convert this value to a percentage on a moist mass ("as is") basis, Equation 3.3 was used.

$$\%HS_{\text{as is}} = \frac{V_{extr} \times C_{HS}}{V_{inj} \times 10^6 \times m_{org}} \times \frac{100}{1} \qquad \ldots 3.3$$

where: V_{extr} is the volume of 70% MeOH-H_2O added to the dry sample (µl)

C_{HS} is the concentration of HS from the regression equation (µg/10µl)

V_{inj} is the volume of extract injected into the HPLC (µl)

m_{org} is the original mass of the dry sample (g)

The HS content on a dry mass basis ($\%HS_{DB}$) was also calculated using Equation 3.3 with the m_{org} value replaced by m_{adj}, calculated as follows (Equation 3.4):

$$m_{adj} = m_{org} - \left(\frac{M_{ave}}{100} \times m_{org} \right) \qquad \ldots 3.4$$

where: m_{adj} is the adjusted mass of the sample (g)

m_{org} is the original moist mass of the sample (g)

M_{ave} is the average moisture content of the sample (%)

Table 3.2 Dilution series for harpagoside standard solutions (concentration of the stock solution = 1.94 $\mu g.\mu l^{-1}$).

Vol_{Stock}* (μl)	Concentration ($\mu g.10\ \mu l^{-1}$)	Average area ± SD† ($\mu V.s$)
25	0.524	2116474.0 ± 6089.6
50	1.021	4090412.5 ± 31939.9
75	1.492	5920848.5 ± 69819.0
100	1.940	7555677.0 ± 43686.5
125	2.366	9404637.0 ± 50866.4

* Vol_{stock} = volume (in μl) of the original stock solution used, each diluted with 900 μl methanol.
† Average area ± SD = average integration response (in $\mu V.s$) between duplicate HPLC injections ± standard deviation.

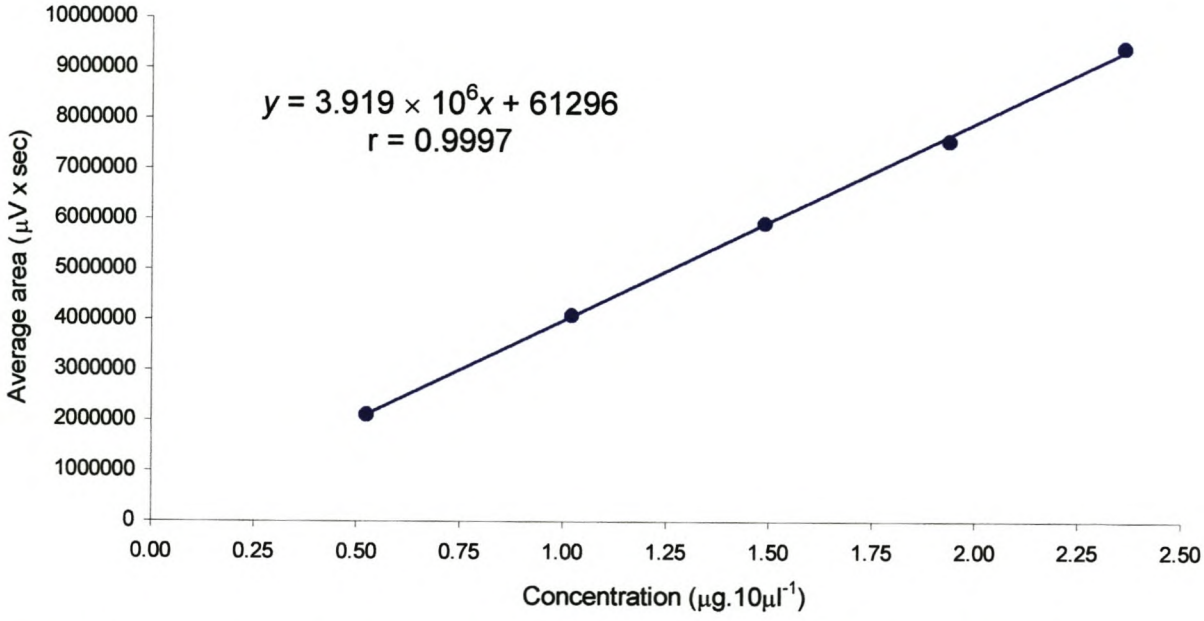


Figure 3.1 Standard curve for harpagoside determination in Devil's Claw root.

For comparative purposes, the $\%HS_{DB}$ content was used in order to compensate for small differences in the MC of individual samples.

Statistical analyses

Data for statistical analysis was imported from MS-Excel 2000 spreadsheets into the SAS (version 6.12) software package. Statistical comparisons were done after analysis of variance, where replicates were treated as blocks, and Student's t-LSD test (significance level = 0.05) were performed.

Results and Discussion

Samples

The 70% MeOH-H_2O extracts showed slight precipitation during cold storage. This is believed to have been caused by extracted plant waxes, probably ursolic acid and its isomer oleanolic acid, as well as 3-β-acetyloleanolic acid (Ragusa *et al.*, 1984; Duke, 2000; Majeed & Nujoma, 2000). However, if the extracts were left to reach ambient temperature, no precipitate was evident. Problems with peak broadening during the HPLC analyses and the subsequent loss of resolution that prompted frequent replacement of the guard column, could be the result of these waxes and other compounds precipitating on the guard column.

HPLC measurements

The HPLC method of Feistel & Gaedcke (2000) was modified to overcome the occurrence of large pressure fluctuations at the end of the program. Lengthening the program from 60 minutes to 70 minutes allowed better equilibration of the column with the aqueous phase before the next injection. A typical chromatogram is given in Figure 3.2, with HS eluting at *ca.* 40.3 minutes.

The large range of solvents used in other studies (Lanhers *et al.*, 1992; Guillerault *et al.*, 1994; Baghdikian *et al.*, 1997) for the extraction of HS, prompted the testing of two solvents with different polarities, namely 70% MeOH-H_2O and 100% MeOH. HPLC analyses revealed significant ($P < 0.05$) differences between these solvents, with 70% MeOH-H_2O giving better extraction of HS. A summary of the extraction data is given in Table 3.3. According to Ziller & Franz (1979) the water soluble fraction of an extract of *H. procumbens* indicated the presence of the major iridoid (HS). This implied at least partial extraction of HS with water and it was decided to standardise the method on 70% MeOH-H_2O as solvent.

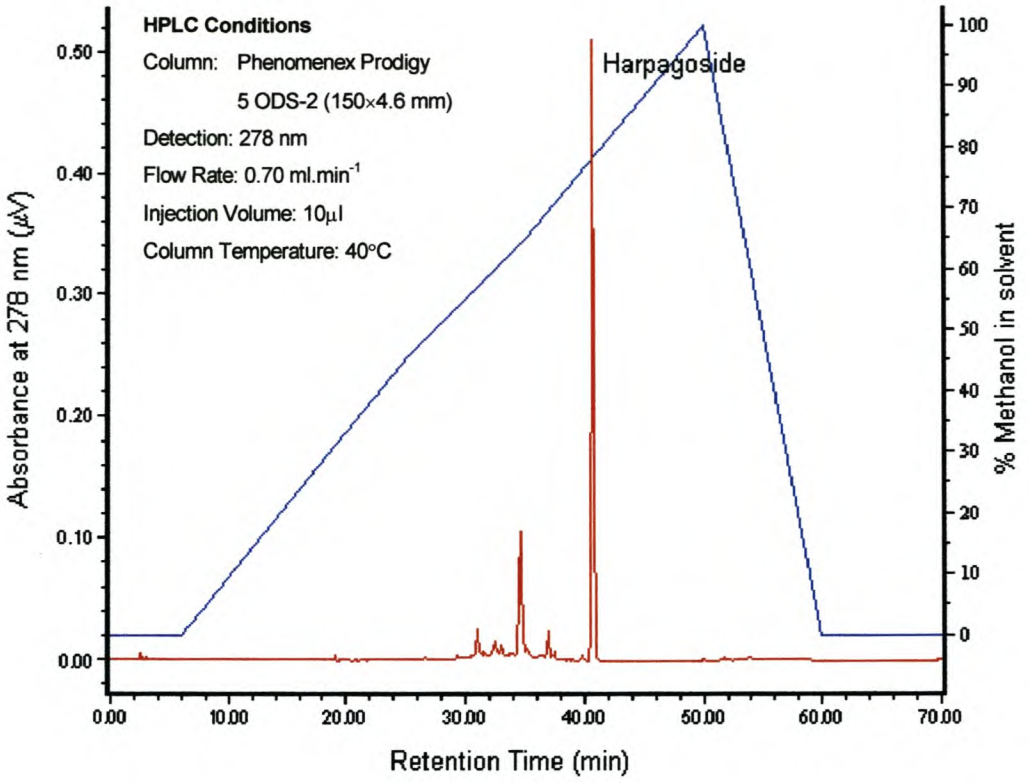


Figure 3.2 Typical HPLC chromatogram (—) of *Harpagophytum procumbens* extract (70% MeOH-H_2O) with program conditions and changes in methanol gradient shown (—).

Table 3.3 Comparison of harpagoside extraction with 100% methanol and 70% methanol, respectively.

Sample Number	HS_{100}* (µg. 10 µl^{-1})	HS_{70}† (µg. 10 µl^{-1})	$\frac{HS_{100}}{HS_{70}} \times 100\%$ ‡
1	1.207	1.375	87.79
2	1.085	1.183	91.76
3	1.020	1.097	92.98
4	1.924	2.128	90.45
5	2.050	2.380	86.13
6	0.972	1.085	89.58
7	0.970	1.084	89.41
8	1.061	1.179	90.03
9	1.094	1.150	95.16
10	1.275	1.344	94.85
11	1.373	1.387	98.97
12	1.055	1.143	92.33
Average	1.257	1.378	91.62

* HS_{100} = harpagoside concentration (in µg.10µl^{-1}) calculated for the 100% methanol extract.
† HS_{70} = harpagoside concentration (in µg.10µl^{-1}) calculated for the 70% methanol-water extract.
‡ ratio between HS_{100} and HS_{70}, expressed as a percentage.

The multiple extraction test with 70% MeOH-H_2O indicated an initial extraction of *ca.* 91% for HS (Table 3.4) that was considered sufficient for analyses and comparative studies. It was assumed that the sum of the HS content for the first and second extractions equalled 100%.

Comparison of drying method

The freeze-dried samples retained the original shape of the root disks (little or no wrinkling) and a pale yellow colour very similar to that of the fresh root (Figure 3.3). Both the sun (Figure 3.4) and tunnel-dried (Figure 3.5) samples shrunk and became twisted during drying. Light to dark brown discolouration occurred with the sun-dried samples having the darkest colour.

Sun-drying required 3 to 5 days per sample, depending upon the prevalent weather conditions. The overall average temperatures (24 h) across all days ranged from 17.55°C to 25.91°C (mean ± SD: 21.82°C ± 2.29°C), with RH values varying between 53.11% and 81.31% (70.09% ± 9.58%). The sun-dried samples had the highest dry MC (6.17% ± 0.72%) of the different drying methods. This may be explained by the relatively low average drying temperatures, compared to the higher tunnel temperatures. The larger the difference between the ambient and the drying temperature, the larger the driving force for removing the water (Hallström & Skjöldebrand, 1983). A further factor was the much higher RH values that would also have reduced the drying potential of this method. Tunnel-dried samples had the lowest MC (3.43% ± 0.66%) due to the higher drying temperatures and lower RH (30%) used. Freeze-drying (5.08% ± 1.05%) was less effective than tunnel-drying. The average moisture and harpagoside contents are given in Table 3.5 (results for individual samples are given in Addendum A).

Sun-drying resulted in significantly ($P < 0.05$) lower HS concentrations (1.455% ± 0.362%) than the other two methods. Even though freeze-drying (1.565% ± 0.394%) and tunnel-drying (1.526% ± 0.396%) did not differ significantly ($P > 0.05$), the observed trend indicated that freeze-drying would be most beneficial for HS retention (Figure 3.6). The large standard deviation for all of the drying methods reflects the relatively large HS range across the 8 replicates (1.098% - 2.082% for the sun-dried samples) that is indicative of the inter-root harpagoside variation (Czygan & Krüger, 1977; Eich *et al.*, 1998; Feistel & Gaedcke, 2000).

It is possible that the greater decrease in HS content during sun-drying can partially be attributed to the long drying time. Slow drying, due to the high ambient RH values (*ca.* 70%), could have increased the incidence and degree of enzymatic degradation, by maintaining the water activity (a_w) above 0.7 for an extended period of time (Rockland & Nishi, 1980). Under these conditions, the initial stages of drying would be characterised by higher levels of free water in the

Table 3.4 Summary of the multiple extraction of Devil's Claw with 70% methanol-water.

Sample Number	HS_1* ($\mu g.\ 10\ \mu l^{-1}$)	HS_2† ($\mu g.\ 10\ \mu l^{-1}$)	$\left(\frac{HS_1}{HS_1 + HS_2}\right) \times 100\%$‡
1	1.001	0.083	92.33
2	1.173	0.109	91.47
3	1.224	0.103	92.23
4	1.735	0.152	91.94
5	1.490	0.146	91.09
6	1.249	0.107	92.12
7	1.868	0.164	91.91
8	1.268	0.116	91.65
9	1.465	0.138	91.36
10	1.140	0.113	90.97
11	1.346	0.119	91.88
12	1.329	0.125	91.43
Average	1.357	0.123	91.70

* HS_1 = harpagoside concentration (in $\mu g.10\mu l^{-1}$) calculated for the first extraction period.
† HS_2 = harpagoside concentration (in $\mu g.10\mu l^{-1}$) calculated for the second extraction period.
‡ percentage initial extraction, assuming (HS_1 + HS_2) equals 100%.

Figure 3.3 Freeze-dried Devil's Claw (*Harpagophytum procumbens*) root, cut into 6 mm disks before drying.

Figure 3.4 Sun-dried Devil's Claw (*Harpagophytum procumbens*) root, cut into 6 mm disks before drying.

Figure 3.5 Tunnel-dried Devil's Claw (*Harpagophytum procumbens*) root, cut into 6 mm disks before drying.

Table 3.5 Average moisture and harpagoside (HS) contents (mean ± standard deviation) for sun, tunnel and freeze-dried Devil's Claw root. The roots were cut into 6 mm disks and divided between the drying methods. Tunnel conditions were set at 60°C and 30% relative humidity.

Drying method	% Moisture	$\%HS_{as\ is}$ *	$\%HS_{DB}$ †
Sun	6.17 ± 0.72a‡	1.366 ± 0.341a	1.455 ± 0.362a
Tunnel	3.43 ± 0.66b	1.475 ± 0.389b	1.526 ± 0.396b
Freeze	5.08 ± 1.05c	1.483 ± 0.362b	1.565 ± 0.394b

* $\%HS_{as\ is}$ = harpagoside concentration expressed on a 'wet mass' basis.
† $\%HS_{DB}$ = harpagoside concentration corrected for moisture (dry basis).
‡ Values within the same column with different letters are significantly different ($P < 0.05$); Student's t-LSD test with replicates treated as blocks.

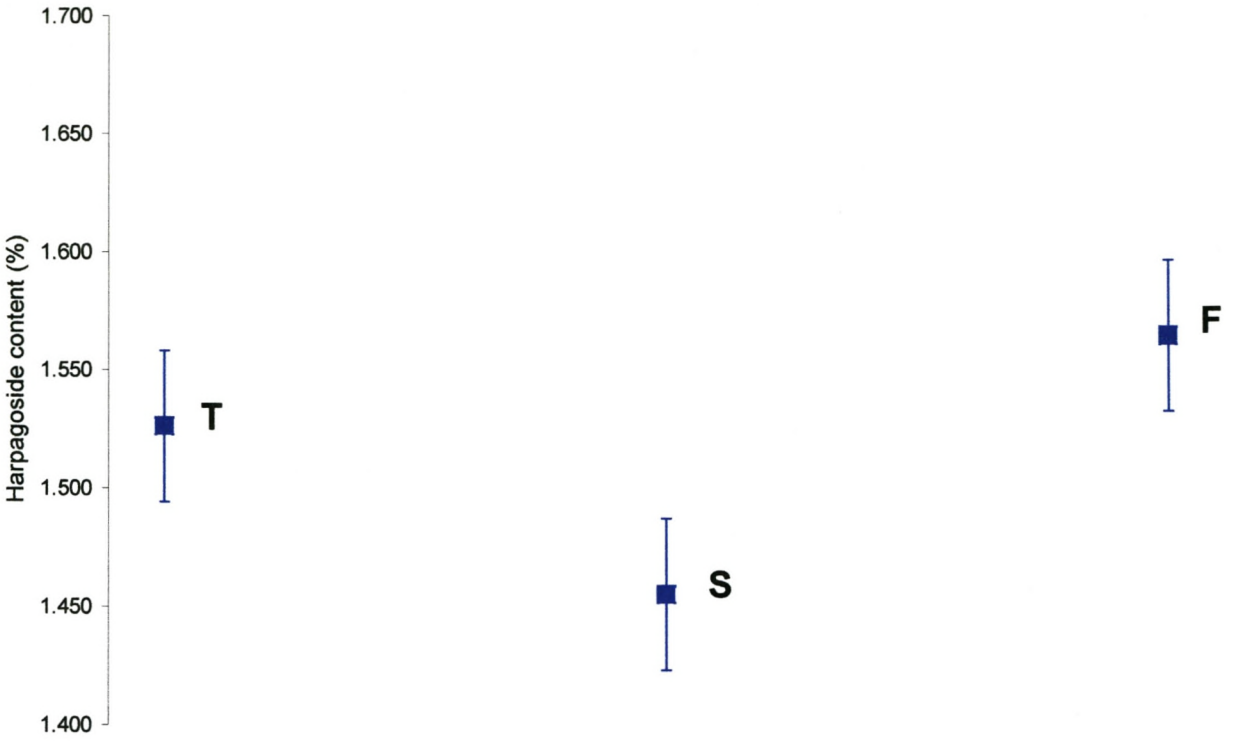


Figure 3.6 Comparison of three drying methods (T = tunnel-drying, S = sun-drying, F = freeze-drying) for the retention of harpagoside (HS) in Devil's Claw root. The 95% confidence interval of HS for each drying method is given.

root; a prerequisite for enzymatic reactions. The ambient drying temperatures (average: 21.82°C) would also be within the optimum range of many such enzymes. Specifically, the activity of polyphenol oxidase (PPO) and glucosidases have been implicated in degrading reactions in plants (Conn, 1993; Vámos-Vigyázó, 1995).

Freeze-drying was expected to be the least detrimental drying technique because of the low drying temperature and the fact that reduced pressure caused the water component to change directly from a solid to a vapour phase (i.e. sublimation). No water, in a liquid state, would be available for chemical reactions under these conditions. Additionally, drying under vacuum limited the availability of oxygen for enzymatic reactions. Such reactions, especially the action of PPO, can cause browning of a variety of fruits and vegetables (Vámos-Vigyázó, 1995; Walker, 1995). Polyphenol oxidase, also known as catechol oxidase or phenolase, has been identified in almost all higher plants (Lax & Cary, 1995; Sherman *at al.*, 1995). Its activity is especially prevalent in the browning of apples and potatoes (Sapers *et al.*, 1995; Wakayama, 1995). The lack of extensive discolouration in the freeze-dried samples (Figure 3.3) indicates that neither such enzymatic, nor non-enzymatic chemical reactions due to high drying temperatures, occurred.

Operating a freeze-drier for commercial drying purposes could, however, prove difficult and costly (Lorentzen, 1983), and from the data it can be concluded that tunnel-drying would still produce an acceptable product (minimal decrease in HS content compared to freeze-drying) at a lower cost.

Drying curves

Drying curves were generated for the three tunnel-drying temperatures, i.e. T_{DB} = 40°C, 50°C and 60°C at 30% RH. The main purpose of such curves is the modelling of the drier and the parameters of drying. This becomes important in the design of a drier, especially if product-specific drying is required (Diamante & Munro, 1991). One of the important drying parameters required for this study was the termination time that would produce samples of similar moisture content for each drying temperature.

Figure 3.7 shows the moisture content (wet basis) plotted against drying time at 40°C, 50°C and 60°C. The three curves were normalised by using an average (n = 18) solids content of 12.73%. From Figure 3.7 it was possible to estimate the drying time required that would produce products of *ca.* 7% MC. This desired MC was selected to ensure that over-drying did not occur. Commercially, over-drying would be undesirable from both a quality and production point of view, where slightly higher moisture losses would not compensate for the incurred costs of a

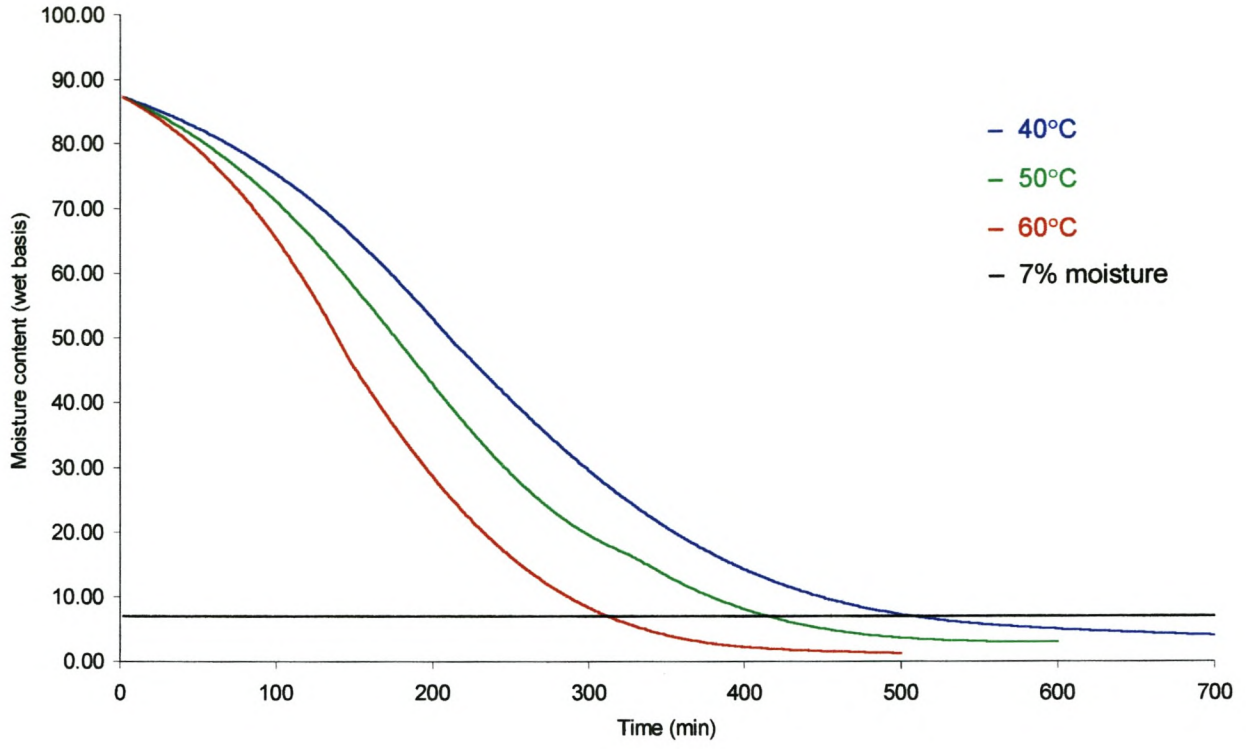


Figure 3.7 The percentage moisture content (wet basis) of Devil's Claw samples (6 mm disks), measured at three dry bulb temperatures, as a function of drying time. A small-scale, experimental tunnel-drier was used with the air humidity set at 30% and an air flow of 2.0 $m.s^{-1}$.

prolonged drying period. From a quality perspective, it could cause serious losses of the active components, while insufficient drying would leave the product moist and susceptible to microbial spoilage.

Initially, the calculated drying times were *ca.* 9 hours, 7 hours and 5 hours for 40°C, 50°C and 60°C, respectively. However, trials in the larger tunnels showed these drying times to be too short, especially for the lower drying temperatures. Subsequently, they were adjusted to 17 hours, 11 hours and 6 hours, respectively. Even though the sample size increased to *ca.* 3 times the amount used in the small-scale drier, the initial drying times should have been adequate in the larger tunnels, especially due to the increase in air flow rates and the fact that the RH remained constant at 30%. The roots used for this experiment were, however, harvested much later than those used in the small-scale drier. It is possible that changes in the cellular structure of the roots, due to seasonal influences, could have increased the water binding capacity of the samples. Stachyose, a tetrasaccharide, is abundant in the root with up to 46% occurring in dried material (Ziller & Franz, 1979). This oligosaccharide, possibly functioning as a reserve carbohydrate, could conceivably increase during winter and afford a higher concentration of polymers in older roots. A higher sugar concentration would suppress the a_w (i.e. free water) in the root and, subsequently, increase the rate of mass transfer and the drying time required (Mazza, 1982). It was also observed that roots dried at 60°C remained malleable until removed from the drier. The malleability of the product made it difficult to judge when the product was adequately dried and this uncertainty could have caused an additional increase in the drying time.

A plot of the drying rate of a sample as a function of its moisture content is depicted in Figure 3.8. Two drying rate periods are clearly visible on the plot, namely the induction and falling rate periods. No clear constant rate period was obtained, because for this to occur, the rate of migration of water from the core of the sample to the exterior should be fast enough to maintain a wet surface (McCormick, 1979). Even though Devil's Claw had a high moisture content in the fresh root (*ca.* 87%), the high air velocities in the drying tunnels (3 - 4 $m.s^{-1}$) and the relatively large surface area of the 6 mm disks would have resulted in an evaporation rate, or rate of heat transfer, higher than the rate of mass transfer (Chirife & Cachero, 1970; Hallström & Skjöldebrand, 1983). A further explanation for the lack of constant drying rates may be found in the relatively large temperature differentials at a constant 30% RH, i.e. 13.91°C for T_{DB} = 40°C, 17.68°C for T_{DB} = 50°C and 20.28°C for T_{DB} = 60°C. Assuming that the surface temperature of the wet product approximated the wet bulb temperature (T_{WB}), the rate of evaporation obtained for these temperature differentials would have been rapid enough to prevent constant rate drying.

The induction period is characterised by an increase in sample temperature, approaching

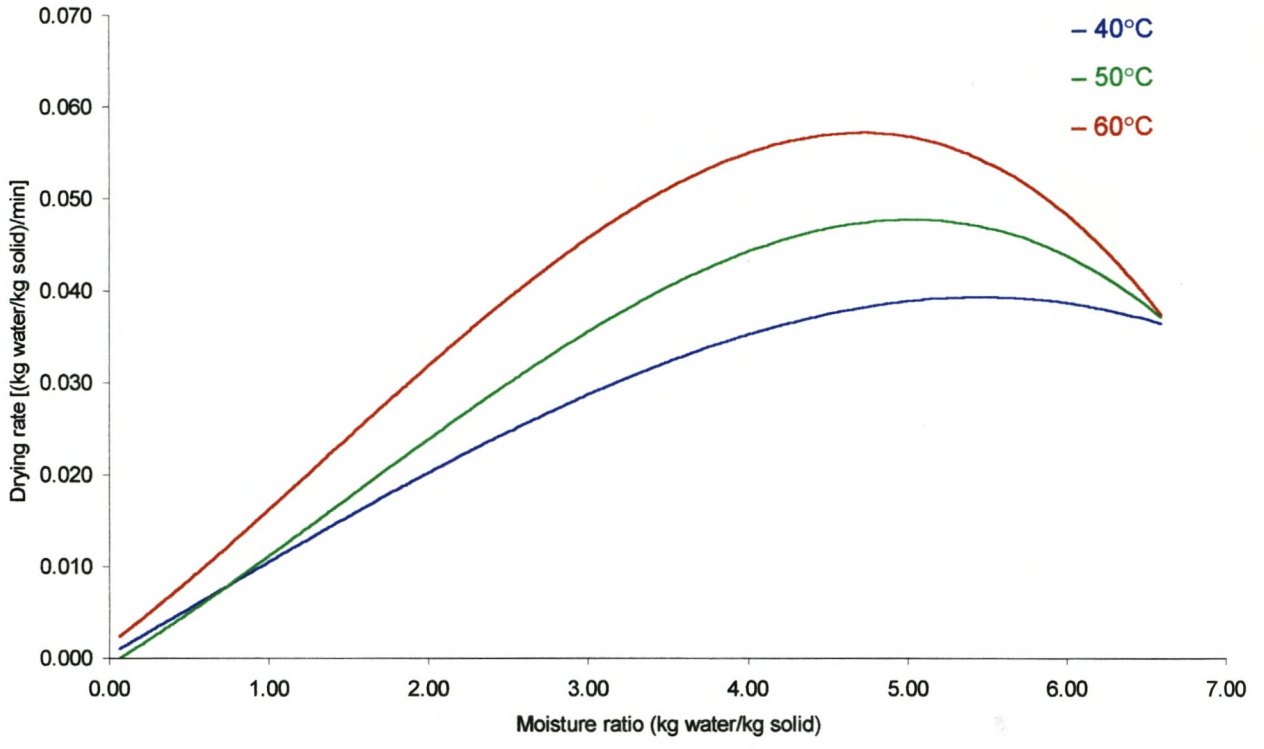


Figure 3.8 The drying rate of Devil's Claw samples (6 mm disks), measured at three dry bulb temperatures, as a function of the moisture content (dry basis) during drying. A small-scale, experimental tunnel-drier was used with the air humidity set at 30% and an air flow of 2.0 $m.s^{-1}$.

the T_{WB}. The induction period for 40°C was relative short, with only a slight increase in drying rate, as a result of the low T_{WB} (26.09°C). Even though no clearly discernable constant rate drying was observed for any of the temperatures, drying at 40°C provided the closest approximation to this period. Mild temperatures and a high free water content during this period of drying are ideal for possible enzyme reactions to occur (Hallström & Skjöldebrand, 1983). It was, therefore, desirable not to have an extended constant rate drying period, since enzymatic reactions during this period could possibly have a negative impact on the HS content of the root.

Falling rate drying is strongly influenced by internal heat and mass transfer (McCormick, 1979). This implies that some of the factors that influence the constant rate drying, will still have an effect during this period. This is confirmed by the lower drying rate at 40°C; approximately two-thirds of the rate at 60°C measured at a moisture content of *ca.* 3.5 kg water/kg solids (Figure 3.8). The effect of the lower drying rates can also be observed by the extended drying time required for drying at 40°C. The falling rate periods of all three temperatures approximated a linear curve between moisture contents of *ca.* 0.5 to 3.5 kg water/kg solids. This linear decline indicates that no sudden changes occurred in the sample matrix, such as case hardening that would have been characterised by a sudden drop or decrease in the drying rate (Von Loesecke, 1945; Thijssen, 1979).

The increasing temperature of the roots themselves during the falling rate period, due to the continuous decrease in moisture content, would increase the rate of thermal decomposition of heat-labile compounds (Thijssen, 1979). A balance between temperature and drying time is, therefore, required.

Comparison of drying temperature

The average moisture content of all the samples ($n = 27$) was 8.89% ± 1.10%. The changes in HS content were significantly ($P < 0.05$) different between 40°C and 50°C, but drying at 60°C did not differ significantly ($P > 0.05$) from the either of the other two temperatures. The average moisture and harpagoside contents are given in Table 3.6 (results for individual samples are given in Addendum B). Figure 3.9 indicated that drying at 50°C would be most beneficial for the retention of HS, while the lowest retention would occur at 40°C. The change in HS retention with drying temperature also indicated that the observed differences in drying rates are important.

It is probable that the slow drying at 40°C created a more ideal environment, with high enough water activity for enzymatic reactions to occur, than at either 50°C or 60°C. During the initial stages of drying the sample temperature would remain lower than the T_{DB} (i.e. closer to the

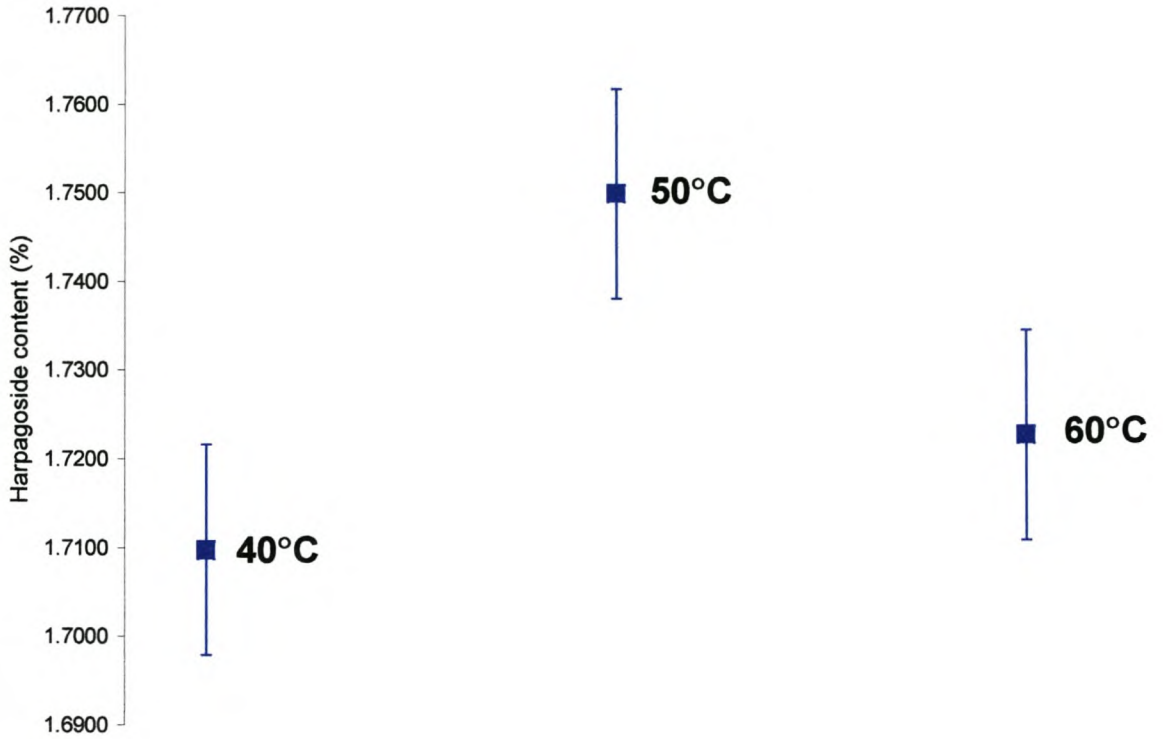


Figure 3.9 Comparison of three tunnel-drying temperatures (40°C, 50°C and 60°C, at 30% relative humidity) for the retention of harpagoside (HS) in Devil's Claw root. The 95% confidence interval of HS for each drying temperature is given.

Table 3.6 Average moisture and harpagoside (HS) contents (mean ± standard deviation) for Devil's Claw root dried in a tunnel at 40°C, 50°C and 60°C and a relative humidity of 30%. The roots were cut into 6 mm disks and divided between the drying temperatures.

Temperature	% Moisture	$\%HS_{as\ is}$ *	$\%HS_{DB}$ †
40°C	9.13 ± 1.16a‡	1.553 ± 0.389a	1.710 ± 0.426a
50°C	8.84 ± 1.00a	1.597 ± 0.406b	1.750 ± 0.438b
60°C	8.71 ± 1.21a	1.573 ± 0.410ab	1.723 ± 0.444ab

* $\%HS_{as\ is}$ = harpagoside concentration expressed on a 'wet mass' basis.
† $\%HS_{DB}$ = harpagoside concentration corrected for moisture (dry basis).
‡ Values within the same column with different letters are significantly different ($P < 0.05$); Student's t-LSD test with replicates treated as blocks.

T_{WB}) and within the optimum range (*ca.* 25°C to 35°C) for enzyme activity. The data would seem to suggest that the evaporation and mass transfer of water from samples dried at 50°C was rapid enough to reduce the incidence of possible enzymatic degradation reactions in the presence of free water. Furthermore, the sample temperature remained low enough so as not to increase the reaction rate of any chemical degradations dramatically. Lower HS content at 60°C than at 50°C could be due to the enhancement of temperature-dependant, non-enzymatic chemical reactions (Petrucci & Harwood, 1993). It is possible that during the induction period for both temperatures the root temperature remained low enough for enzymatic browning intermediates to form (Sapers *et al.*, 1995). These would, however, undergo oxidation or condensation reactions at a faster rate for 60°C drying once the sample temperature increased. Further polymerisation reactions with free amino acids may lead to the production of other intermediates that can possibly cause the reduction of HS content (Ziller & Franz, 1979; Baghdikian *et al.*, 1999).

Besides PPO, β-glucosidase is also prevalent in the plant kingdom. It has been implicated in the activation of phytohormones by hydrolysis of its glucoside storage form and in cyanogenesis (i.e. the release of HCN from cyanogenic glucosides), a defence mechanism in plants (Esen, 1993). The stability of β-glucosidase has also been shown in the presence of a variety of denaturing agents and can be said to approximate thermophilic enzyme activity (Esen & Gungor, 1993). All of the iridoids in Devil's Claw are β-glucosides and are therefore susceptible to hydrolysis by this enzyme. The thermophilic nature of β-glucosidase poses a problem since elevated drying temperatures would probably have less effect than on PPO. Further study is required to determine whether the hydrolysis of HS by β-glucosidase might produce a medicinally active form in the plant.

The production of coloured polymers, and the subsequent decrease of HS, may also be the result of the acid lability of the aglycone moiety of HS (Burger, 1985). A localised acidic environment could possibly be created during high-temperature drying, if the thermal or enzymatic degradation of plant waxes were to release fatty acids. This would, however, not necessarily reduce the medicinal potency of the root, as certain studies have shown the possibility of gastric acid inducing the hydrolysis of HS to form harpagogenine, possibly the active form of HS (Haag-Berrurier *et al.*, 1978; Mills & Bone, 2000; Loew *et al.*, 2001). It is, however, debateable whether released fatty acids could create an environment with high enough acidity for hydrolysis to occur.

Browning occurred in all samples, irrespective of the drying temperature used and PPO could be responsible for enzymatic browning. Typically, PPO has an optimum activity at

Figure 3.10 Selected sun and tunnel-dried Devil's Claw (*Harpagophytum procumbens*) root with evident green to blue-black discolouration of the cortical zone (indicated by the arrows).

ca. 30°C, with inactivation occurring rapidly above temperatures of 60°C (Lee-Kim *et al.*, 1995; Wakayama, 1995). The mechanism of browning therefore requires mild temperatures and the availability of oxygen for the oxidation of phenolic compounds to quinones that, in turn, form pigmented melanins (Lee-Kim *et al.*, 1995; Whitaker & Lee, 1995).

Although no measure of quality is associated directly with the colour of the dried root, it can be inferred that a large degree of browning might negatively influence the consumer perception of the product. Freeze-drying does not present this problem, but it is expensive to use. During tunnel-drying or sun-drying, a more distinct colour change is apparent, but is probably not sufficient to prove detrimental from a visual aspect. Certain samples did, however, show a green to blue-black discolouration in the cortical zone of the disks (Figure 3.10) that may indicate polymerisation reactions or the lability of the aglycone moiety (Burger, 1985).

Conclusion

The results of this study showed that the conditions used for the drying of *Harpagophytum procumbens* root are important for the harpagoside retention in the dried product. The influence of the drying method on HS retention showed significant results and a strong trend in the order freeze-drying > tunnel-drying > sun-drying. Although this trend suggests that freeze-drying would be the best method to use, the costs incurred for the maintenance of such a system could prove too high in a commercial situation. Consequently, tunnel-drying is suggested as the best alternative.

The comparison of drying temperatures indicated that drying at 50°C (dry bulb) and a 30% RH would prove most beneficial to the retention of HS. Both the enzymatic and non-enzymatic reactions could also be causal in the relatively lower HS content values obtained with drying at 40°C and 60°C, as they seem to be linked to the browning of the root. The browning may be evidence of deterioration reactions that can reduce HS content. For similar reasons, sun-drying is not recommended as a drying method, unless it is the only available option.

For tunnel-drying, the exact drying conditions used in practice, would depend largely on the particular drier available. A combination of the drying temperature and time required for cost-efficient operation would determine the most suitable conditions, and further study would be required to optimise these.

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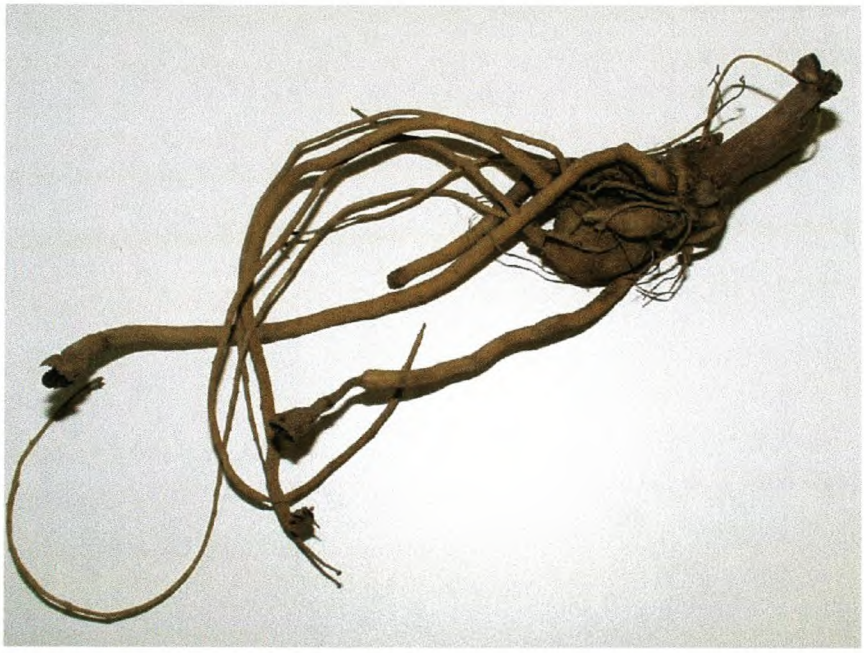
Chapter 4

Application of near infrared spectroscopy (NIRS) for determination of the harpagoside, 8-ρ-coumaroyl harpagide and moisture contents, and harpagoside:8-ρ-coumaroyl harpagide ratio in dried *Harpagophytum procumbens* root



Chapter 4

Application of near infrared spectroscopy (NIRS) for determination of the harpagoside, 8-ρ-coumaroyl harpagide and moisture contents, and harpagoside:8-ρ-coumaroyl harpagide ratio in dried *Harpagophytum procumbens* root

Abstract

The prediction, by near infrared spectroscopy (NIRS), of harpagoside (HS), 8-ρ-coumaroyl harpagide (8ρCHG) and moisture contents (MC), as well as the ratio between HS and 8ρCHG, in dried, ground Devil's Claw (*Harpagophytum procumbens*) root was evaluated. In all cases, partial least squares (PLS) regression was used to develop calibrations from spectra generated by either Perkin-Elmer Spectrum IdentiCheck FT-NIR or Foss NIRSystems 6500 spectrophotometers. The relative performance of these instruments was also evaluated. Pre-processing of spectra involved various combinations of multiplicative scatter correction (MSC) and first or second derivatives (Savitsky-Golay). Two data sets (containing 150 and 42 samples, respectively) were used and the predictive abilities of the developed models were tested either by independent validation or, in those cases where the number of available samples was limiting, full cross-validation. Excellent results were obtained from the FT-NIR MC calibration (range: 2.44% to 10.43%), with a standard error of prediction (SEP) and correlation coefficient (*r*) of 0.24% and 0.99, respectively. The standard error of laboratory (SEL) for the MC reference method was calculated as 0.14%. The best HS calibration, with SEP and *r* values of 0.134% and 0.90, was obtained from NIRSystems spectra. Similarly, the best 8ρCHG calibration, with a standard error of cross-validation (SECV) of 0.028% and *r* of 0.91, was also obtained from models using these spectra. The SEL values for the HS and 8ρCHG reference methods were calculated as 0.035% and 0.007%, respectively. The HS and 8ρCHG models could be used for screening purposes to classify Devil's Claw root into low, middle or high HS content classes. Such a classification system would allow for standardisation of the export quality of the product and remunerations to producers. The calibrations using the HS:8ρCHG ratio as reference were evaluated for distinction between the species, *H. procumbens* and *H. zeyheri*, but contrary to previous studies using this parameter, the separation between species was not considered large enough to use this model for the intended purpose. Only two authenticated *H. zeyheri* samples could be included in the data sets and future studies should involve a larger number of confirmed *H. zeyheri* samples. Principle component analysis (PCA) of the spectral data, however, revealed clear groupings between the species. This shows great potential for the future authentication of Devil's Claw species.

Introduction

Harpagophytum procumbens, commonly known as Devil's Claw, is a prostrate, perennial herb, indigenous to Southern Africa (Watt & Breyer-Brandwijk, 1962). The tuberous secondary roots are purported to possess anti-inflammatory and analgesic activity (Watt & Breyer-Brandwijk, 1962; Eichler & Koch, 1970; Baghdikian *et al.*, 1997). It has been suggested that a second species, *H. zeyheri*, shows similar activity (Czygan & Krüger, 1977; Baghdikian *et al.*, 1997), although it is generally accepted that only *H. procumbens* is effective (Eich *et al.*, 1998; Chrubasik *et al.*, 2000). The roots are typically harvested by hand, sliced and then sun or tunnel-dried and exported without further processing. The dried product can then be ground to a powder which may be infused to produce a herbal tea or alcoholic extract (G. Hansford, Grassroots Natural Products, Gouda, South Africa, personal communication, 2001). The medicinal properties of these roots have been linked to its iridoid glucoside content and in particular to the iridoid, harpagoside (HS) (Guillerault *et al.*, 1994; Baghdikian *et al.*, 1997).

When dealing with herbal products, large variations in active compounds are often found naturally. The distribution and retention of such compounds in the final product can also be greatly influenced by the processing method, especially if this is largely uncontrolled (George *et al.*, 2001). If, for example, the product requires drying, lack of proper control could introduce larger variation in the active compounds of the final product than there had been naturally, as a result of physico-chemical changes occurring (Hallström & Skjöldebrand, 1983). A factor of further importance to such herbal products, both due to its influence on storage stability and microbial growth and the inherent economic implications, is moisture content (Hall *et al.*, 1988; Ren & Chen, 1997). Quality control during processing and of the final product often calls for tests that can be lengthy, expensive and may involve the use of large quantities of various hazardous chemicals. There is a need to reduce both the analysis time and the environmental impact of these tests, while maintaining a sufficient level of sensitivity.

Usually high-performance liquid chromatography (HPLC) analyses have been used to determine the iridoid content of the root (Guillerault *et al.*, 1994; Feistel & Gaedcke, 2000). The ratio between HS and a second iridoid, 8-ρ-coumaroyl harpagide (8ρCHG) that is not directly linked to any medicinal activity, may be used to distinguish between the two species (Baghdikian *et al.*, 1997; Eich *et al.*, 1998).

If the level of the measured analyte is sufficient, very good quantitative analyses by near infrared spectroscopy (NIRS) may be achieved (Ren & Chen, 1999; Schulz *et al.*, 1999; Steuer *et al.*, 2000). In cases where these levels are close to the detection limit of the NIRS system, calibrations could still be developed as a screening method within set margins (Hall *et al.*, 1988).

The export of a product is often governed by minimum or maximum acceptable levels of particular compounds. In the case of Devil's Claw, if the HS content is too low the successful export of the root becomes problematic, with buyers claiming adulteration of the product (G. Hansford, Grassroots Natural Products, Gouda, South Africa, personal communication, 2001). An accurate and rapid screening method, that may be applied before export, could provide information of economic benefit to the producer and could also standardise the cost to the consumer.

The development of any of these calibrations is usually based on other established analytical methods such as HPLC, mass spectrometry (MS) or gas chromatography (GC). These methods are more sensitive than NIRS, but they often lack the rapid throughput of the latter (Osborne *et al.*, 1993).

NIRS is particularly suited to applications in the food sector (Wetzel, 1998). Some of these include produce or products as diverse as wheat (Ghaedian & Wehling, 1997; Wesley *et al.*, 1999; Cozzolino *et al.*, 2000), wine (Gishen & Dambergs, 1998; Manley *et al.*, 2001), meat (Rannou & Downey, 1997; Pink *et al.*, 1999), edible oils (Sato *et al.*, 1998; Che Man & Setiowaty, 1999; Moh *et al.*, 1999; Velasco *et al.*, 1999) and dairy (Rodriguez-Otero *et al.*, 1997). It has also found application within the pharmaceutical and drug analysis sectors of the medical community (Ren & Chen, 1999). In particular, the analysis of hypericin in *Hypericum perforatum* (St. John's Wort) extracts (Huck, 2002), ginsenosides in *Panax quinquefolium* (American ginseng) extracts (Ren & Chen, 1999) and polyphenols and alkaloids in *Camellia sinensis* (green tea) (Schulz *et. al*, 1999) have been successful.

The aim of this study was to test the ability of NIRS calibrations to predict the harpagoside and 8-ρ-coumaroyl harpagide contents in dried, ground *Harpagophytum procumbens* root. Additionally, both HS:8ρCHG ratio and moisture content (MC) calibrations were also tested.

Materials and Methods

Preparation of samples

Fresh *H. procumbens* secondary roots from cultivated plants were obtained in three batches from Grassroots Natural Products (Plant Improvement Centre, Gouda, South Africa). The first batch (March 2001) contained *ca.* 6 kg of fresh roots and the two subsequent batches (July 2001 and November 2001) 17 kg and 25 kg, respectively. Additionally, dried roots from Namibia were obtained from Coetzee & Coetzee Distributors (Kuils River, South Africa). A small sample of ground *H. zeyheri* (confirmed by thin layer chromatography at Grassroots Natural Products) was also obtained from Namibia.

Three drying methods were used to dry the fresh roots, namely freeze-drying, tunnel-drying and sun-drying. Processing, grinding and storage of samples before analysis were done as described in Chapter 3. In addition to the 75 samples used for analyses in Chapter 3, a further 75 samples were prepared for use in the near infrared (NIR) calibrations.

Extraction

Crude extracts for HPLC analysis of both *Harpagophytum procumbens* and *H. zeyheri* samples were prepared as described in Chapter 3. All extracts were prepared and analysed in duplicate.

Moisture determination

Moisture contents were determined in duplicate as described in Chapter 3. An average of 5.197 g ± 0.555 g (mean ± SD) of each dried, ground sample was analysed.

High-performance liquid chromatography measurements

HPLC measurements were performed as described in Chapter 3, with the additional 75 samples also analysed in duplicate. These remaining samples were either detected at only 278 nm (Sticher & Meier, 1980; Baghdikian *et al.*, 1997; Eich *et al.*, 1998) for HS determinations, or with a combination of 312 nm and 278 nm for detection of 8ρCHG and HS, respectively (Guillerault *et al.*, 1994; Feistel & Gaedcke, 2000). As only a single wavelength detector was available, the HPLC program was set to switch from 312 nm to 278 nm after 38.5 minutes had elapsed. In total, 42 samples were measured under the latter conditions.

Standard solutions

The quantification of HS was described in Chapter 3. Two concentration ranges (0.049 $\mu g.10\mu l^{-1}$ to 0.323 $\mu g.10\mu l^{-1}$; 0.388 $\mu g.10\mu l^{-1}$ to 2.425 $\mu g.10\mu l^{-1}$) were used for quantification of pure 8ρCHG [PhytoLab (Addipharma), Hamburg, Germany]. The solutions were prepared with HPLC grade methanol (Chromasolv®, Merck). As with the HS standard curves the linear regression equations (Figures 4.1 and 4.2) were used to calculate the 8ρCHG concentration in the Devil's Claw samples.

The 8ρCHG concentrations, expressed in $\mu g.10\ \mu l^{-1}$, were converted to $\%8\rho CHG_{as\ is}$ (wet basis) and $\%8\rho CHG_{DB}$ (dry basis) using Equations 3.3 and 3.4 of Chapter 3.

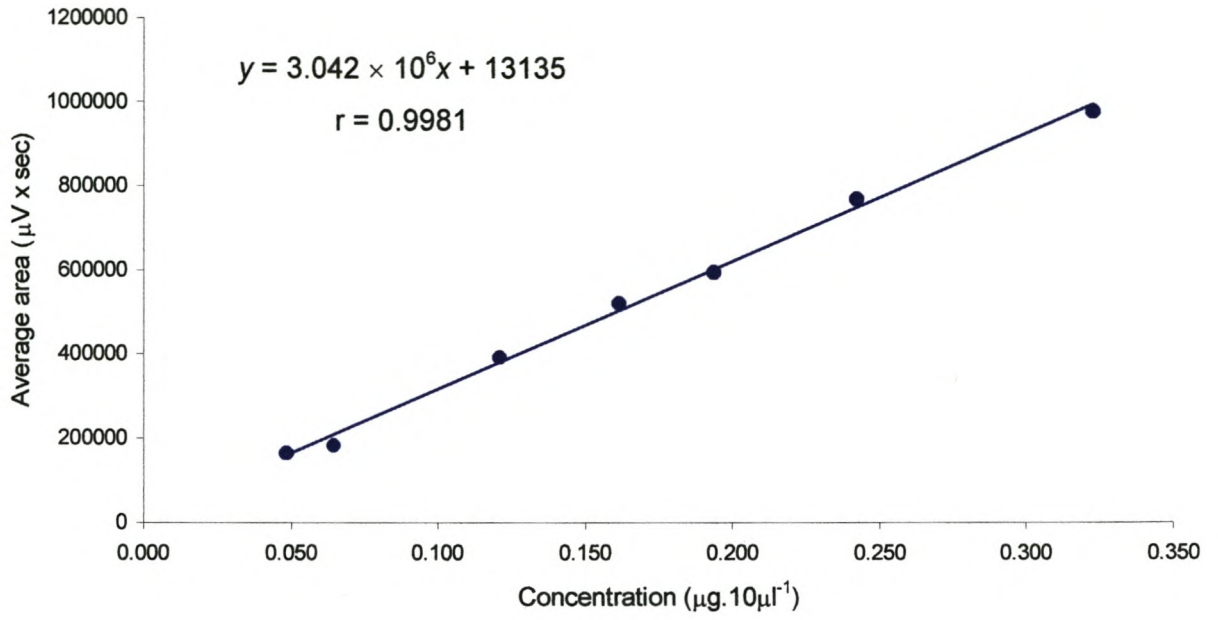


Figure 4.1 Standard curve for 8-ρ-coumaroyl harpagide determination in extracts of dried *Harpagophytum procumbens* root (low concentrations).

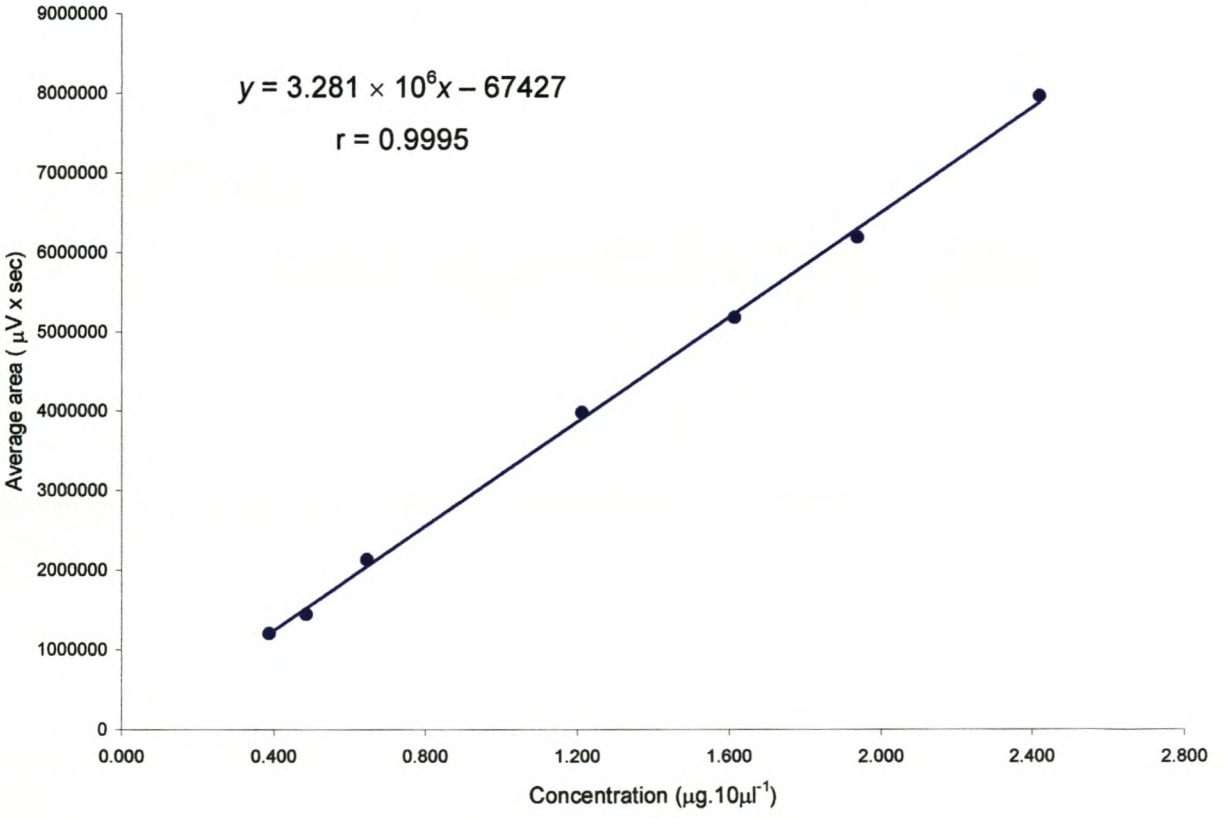


Figure 4.2 Standard curve for 8-ρ-coumaroyl harpagide determination in extracts of dried *Harpagophytum zeyheri* root (high concentrations).

Near infrared spectroscopy measurements

Perkin-Elmer Fourier transform near infrared spectrophotometer

A Perkin-Elmer FT-NIR Spectrum IdentiCheck instrument with IdentiCheck software (version 3.02.01, Perkin-Elmer Incorporated, UK) was used to collect the near infrared spectra. To obtain diffuse reflectance spectra, the IdentiCheck Reflectance Accessory (ICRA) was fitted to the instrument.

Samples were presented to the instrument in 4 ml Chromacol® borosilicate glass vials with screw caps (SMM Instruments, South Africa). Approximately 3 g of each sample was measured into these vials, shaken and gently tapped twice to ensure the even distribution of particles before placing the vials individually in the beam path on the ICRA. The non-reflective lid of the instrument was closed before scanning commenced. Within 60 minutes of scanning, between 3 g and 8 g of each sample, depending on availability, was weighed into nickel oven pans for the moisture content determination.

Reflectance absorption spectra were measured (16 accumulations) across the wavelength range of 1100 nm to 2500 nm (9090.91 cm^{-1} to 4000 cm^{-1}) with 2 nm intervals and at a resolution of 16 cm^{-1}. This implies the generation of 701 data points for each sample. The particular resolution was selected after comparison between scans at 4 cm^{-1}, 8 cm^{-1}, 16 cm^{-1}, 32 cm^{-1} and 64 cm^{-1} for a single *H. procumbens* sample. Selection was based on efficient operation while maintaining maximal absorption. After scanning, the samples were stored in the Chromacol® vials, in desiccators containing silica gel as desiccant.

Foss NIRSystems 6500 spectrophotometer

A total of 150 sub-samples of Devil's Claw (10 g to 13 g each) were stored in 30 ml plastic screw cap vials and sealed in large plastic containers with anhydrous $CaCl_2$ as desiccant. These were sent to the Institute for Plant Analysis in Germany (BAZ, D-06484 Quedlinburg, Germany) for analysis on a Foss NIRSystems Model 6500 instrument.

The NIRSystems 6500 is a scanning monochromator system with 2 nm scanning intervals and a 10 nm bandwidth. Reflectance measurements of the samples were made from 1100 nm to 2500 nm (9090.91 cm^{-1} to 4000 cm^{-1}), generating 701 data points per sample. Spectral data were recorded and exported to MS-Excel 2000 format for further calibration development and comparison with spectra from the Perkin-Elmer FT-NIR Spectrum IdentiCheck instrument.

Calibration development

By default, the file names of Perkin-Elmer spectra have the format "*.sp". To facilitate the transfer of the spectral data to other software packages, the files were converted to ASCII (*.asc)

format and individual files combined by a Microsoft Visual Basic macro into an MS-Excel 2000 spreadsheet (macro kindly programmed by R.S. Gray). In this format, the spectral data (including the data from the Foss NIRSystems 6500) could be imported into both the Simca-P 8.1 (Umetrics AB, Sweden) and The Unscrambler® 6.11 (CAMO ASA, Norway) software packages for further calibration development. Two main sample data sets were used for calibration development to evaluate and compare the performances of the Fourier-transform and monochromator instruments. The first contained all 150 samples analysed for comparisons based on HS and MC values, while the second was limited to 42 of these samples, for comparison of 8ρCHG content and HS:8ρCHG ratios.

All three of the harpagoside (μg.10 μl^{-1}, $\%HS_{as\ is}$ and $\%HS_{DB}$) and 8-ρ-coumaroyl harpagide (μg.10 μl^{-1}, $\%8\rho CHG_{as\ is}$ and $\%8\rho CHG_{DB}$) concentrations were used as reference values to develop calibrations and then compared, per analyte, using the statistical performance indicators of the multivariate data analysis software. Analyses by The Unscrambler® software provided values for the standard error of prediction (SEP) and root mean square error of prediction (RMSEP) (Equations 4.1 and 4.2). Either of these two values reflect the ability of a calibration to predict accurately and precisely the analyte values of the independent validation set, but SEP is corrected for bias (Naes & Isaksson, 1991). The independent validation sets (usually one-third of the total number of samples used) were selected to represent samples across the entire range of available reference values. Every third sample was chosen from an ascendingly sorted data set of reference values.

$$\text{SEP} = \sqrt{\sum_{i=1}^{n} \frac{(\hat{y}_i - y_i - \text{Bias})^2}{n-1}} \quad \text{and} \quad \text{Bias} = \sum_{i=1}^{n} \frac{(\hat{y}_i - y_i)}{n} \qquad \ldots 4.1$$

where: n is the number of samples in the independent validation set;

$\hat{y}_i$ is the predicted (NIRS) value for the i^{th} sample; and

y_i is the measured (reference) value for the i^{th} sample.

$$\text{RMSEP} = \sqrt{\sum_{i=1}^{n} \frac{(\hat{y}_i - y_i)^2}{n-1}} \qquad \ldots 4.2$$

where: n is the number of samples in the independent validation set;

$\hat{y}_i$ is the predicted (NIRS) value for the i^{th} sample; and

y_i is the measured (reference) value for the i^{th} sample.

A similar value, standard error of cross-validation (SECV, Equation 4.3), was reported for certain calibrations where full cross-validation was necessitated by the small number of samples.

$$\text{SECV} = \sqrt{\sum_{i=1}^{n} \frac{(\hat{y}_i - y_i - \text{Bias})^2}{n-1}} \quad \text{and} \quad \text{Bias} = \sum_{i=1}^{n} \frac{(\hat{y}_i - y_i)}{n} \qquad \ldots 4.3$$

where: n is the number of cross-validated samples in the set;

$\hat{y}_i$ is the predicted (NIRS) value for the i^{th} sample; and

y_i is the measured (reference) value for the i^{th} sample.

Calibrations were developed using one of two regression models, partial least squares (PLS) regression or principle component regression (PCR). The performances of these two models were evaluated after a variety of pre-processing methods had been applied to the data. These included multiplicative scatter correction (MSC) to eliminate variances caused by particle size and 1st or 2nd derivatives (both Savitsky-Golay and Norris types) that enhanced slight spectral differences.

In certain cases, outliers were selected from scatter and outlier plots if the samples were evidently grouped away from an observed trend, and as long as they did not represent the natural extremes of the data set. The removal of these subsequently improved the performance of the models. The performances of the various calibrations were also evaluated by comparison of the statistical indicators to the corresponding standard error of laboratory (SEL) for the respective reference methods (Equation 4.4).

$$\text{SEL} = \sqrt{\frac{\sum_{i=1}^{n} (i_1 - i_2)^2}{2n}} \qquad \ldots 4.4$$

where: i_1 is the first reference measurement (% moisture or analyte);

i_2 is the second (duplicate) reference measurement; and

n is the number of duplicate samples.

For any calibration, the type of regression and the pre-processing of the data aim to reduce the SEP (or RMSEP) to approach the SEL of the reference method. The SEP can never be less than the SEL, because this last value is the bench-mark for the analysis and its inherent errors will carry over into the near infrared calibration.

Only 42 HPLC samples had accurate 8ρCHG determinations measured at 312 nm and, as such, formed a relatively small data set for NIRS calibration. To facilitate the development of calibrations with larger sample sets, the remaining 108 samples analysed at 278 nm were adjusted by a factor calculated from the difference between the peak areas for 8ρCHG standards (n = 6) measured individually at both 278 nm and 312 nm (Table 4.1). Adjustments were made to the integration values using Equation 4.5 and the average ratio between areas from Table 4.1.

$$Area_{adj} = \frac{Area_{278}}{0.5387} \qquad \ldots 4.5$$

where: $Area_{adj}$ is the adjusted area of the sample (μV.sec); and
$Area_{278}$ is the original area of the sample measured at 278 nm (μV.sec).

Calibrations were therefore developed using the adjusted 150-sample data set, as well as the original 42-sample data set. Even though the reduced number of samples in the unadjusted 42-sample data set would necessarily lead to better models, the use of full cross-validation should give an indication of the expected performance for a larger data set with independent validation. Due to the large difference between 8ρCHG values of *H. procumbens* and *H. zeyheri*, the two *H. zeyheri* samples were successively included and excluded from the relevant calibrations within each sample data set.

The NIR spectra of the 51 samples used in comparative drying studies described in Chapter 3, were subjected to principle component analysis (PCA) to evaluate whether NIRS could differentiate between the respective drying treatments. Similarly, PCA plots of both sample data sets were used to determine whether NIRS could potentially differentiate between the two *Harpagophytum* species.

Results and Discussion

Moisture determinations

The average MC range (n = 150) was 2.44% - 10.43% (mean ± SD: 4.47% ± 1.58%). Moisture contents of 10% or less have been recommended for dried Devil's Claw (G. Hansford, Grassroots Natural Products, Gouda, South Africa, personal communication, 2001). Milling, a necessary preparation step before the analysis of samples, was however found to be most effective at moisture contents below 8.0%. The SEL of the reference method was calculated as 0.14% (Table 4.2).

Table 4.1 Ratio of 8-ρ-coumaroyl harpagide standards measured by HPLC at 278 nm and 312 nm, for the adjustment of samples measured only at 278 nm.

Dilution (8ρCHG:MeOH)	**$Area_{278}$ (μV.s)**	**$Area_{312}$ (μV.s)**	$\frac{Area_{278}}{Area_{312}}$
1:24	655649.7	1206927.5	0.5432
1:29	547269.7	977994.0	0.5596
1:49	306468.7	596012.0	0.5142
1:59	278911.6	522092.5	0.5342
1:79	209917.2	393722.5	0.5332
1:199	90361.9	165017.5	0.5476
Average			0.5387

$Area_{278}$ = average integrated area for duplicate samples measured at 278 nm.
$Area_{312}$ = average integrated area for duplicate samples measured at 312 nm.

Table 4.2 Summary of the reference data for moisture, harpagoside (HS) and 8-ρ-coumaroyl harpagide (8ρCHG) contents of dried, ground Devil's Claw root.

	% Moisture (n = 150)	**$\%HS_{as\ is}$† (n = 150)**	**$\%8\rho CHG_{as\ is}$ (n = 40) (excluding‡)**	**$\%8\rho CHG_{as\ is}$ (n = 42) (including‡)**
Range	2.44 – 10.43	0.693 – 2.244	0.069 – 0.290	0.069 – 0.958
Mean	4.47	1.348	0.136	0.175
SD	1.58	0.299	0.065	0.187
SEL	0.14	0.035	0.007	0.007

† = ranges, means and standard deviations for HS and 8pCHG are given on a moist mass ("as is") basis.
‡ = calculated values either including or excluding *H. zeyheri* samples.
SD = standard deviation.
SEL = standard error of laboratory (reference method).

HPLC measurements

The eluting times of both 8ρCHG (*ca.* 36.9 minutes) and HS (*ca.* 40.3 minutes) are shown in a typical chromatogram (Figure 4.3).

Due to the large difference in 8ρCHG concentration between the two *Harpagophytum* species (Baghdikian, 1997; Eich *et al.*, 1998; Feistel & Gaedcke, 2000), two separate calibration curves (7 points each) were created to avoid possible non-linearity at low 8ρCHG concentrations. The standard curves are given in Figures 4.1 and 4.2 for *H. procumbens* and *H. zeyheri*, respectively (results for individual samples are given in Addendum C).

The $\%HS_{as\ is}$ range (n = 150) was 0.693% to 2.244% (mean ± SD: 1.348% ± 0.299%). The $\%8\rho CHG_{as\ is}$ content (n = 42) had a range of 0.069% to 0.290% (excluding *H. zeyheri* samples). The mean value, with standard deviation, was 0.136% ± 0.065%. Ranges, averages and standard deviations for all the measured parameters are given in Table 4.2.

The SEL for HS was determined as 0.035%, and for 8ρCHG as 0.007%. The sensitivity of the HPLC method is clearly shown and it should be noted that these values not only reflect the level of error of the instrument, but that of the extraction process as well. The developed calibrations would have to be measured against these performance levels to evaluate their efficacy.

Calibration development

A section of the NIR spectrum (2260 nm to 2400 nm) of Devil's Claw is shown in Figure 4.4 that was used to evaluate the influence of different scanning resolutions with the FT-NIR instrument. There is clear evidence of the loss of spectral information at 64 cm^{-1}. The added sensitivity of 4 cm^{-1} or 8 cm^{-1} does not add to the spectral data and is most likely due to background noise and, compared to the effect at lower resolutions, an increased interference from the glass base of the sample presentation vial. Even though either 16 cm^{-1} or 32 cm^{-1} resolutions could provide sufficient data, it was decided to use 16 cm^{-1} since the scanning time would not be increased much and there was no noticeable reduction in peak absorptions.

The pre-processing of data differed between moisture content and analyte content calibrations, as well as between HS:8ρCHG ratio calibrations. In general, most calibrations involving powders or analysis of small-particled samples should be subjected to multiplicative scatter correction (MSC) before any further calibration is attempted. This is because particle size often creates the largest variation in the spectrum, even if attempts are made to homogenise the samples (Osborne *et al.*, 1993). This study was no different and for most of the calibrations, using

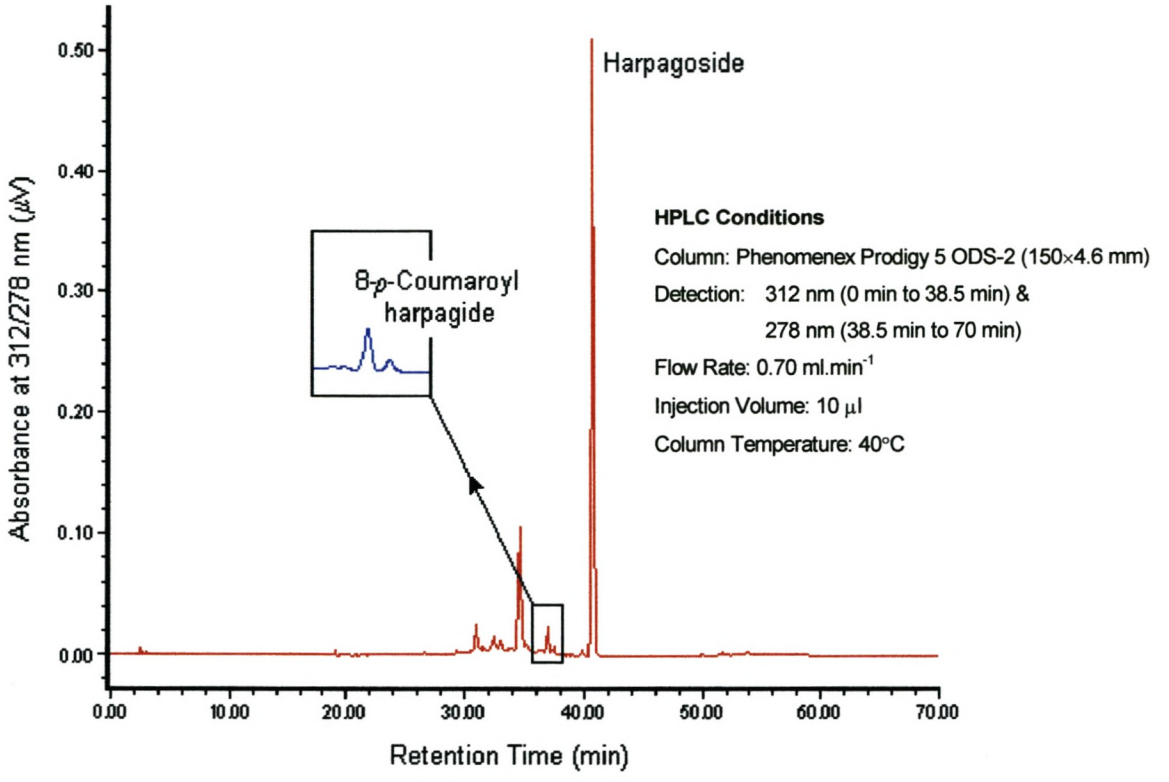


Figure 4.3 Typical HPLC chromatogram of *Harpagophytum procumbens* extract (70% methanol-water) with program conditions.

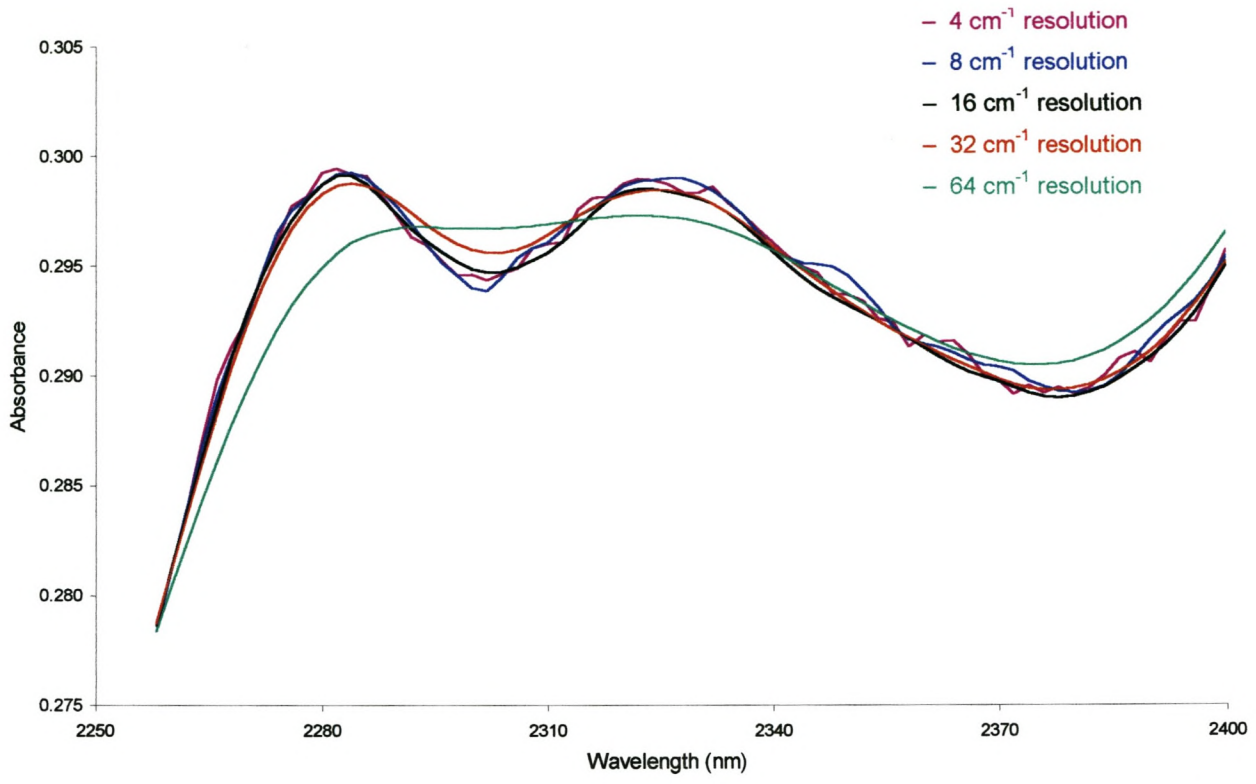


Figure 4.4 Comparison of five resolutions for dried, ground *Harpagophytum procumbens* root scanned on a Perkin-Elmer IdentiCheck FT-NIR instrument.

either analyte or MC reference values, the raw (unprocessed) data provided poorer correlation and predictive ability than data that had been corrected using MSC. It was also found that regression of the data by PLS-1 (single component reference values) provided the best models.

Although all three analyte concentrations were tested, the $\%Analyte_{as\ is}$ values provided the best predictive ability. Calibrations using the $\%Analyte_{DB}$ values led to inferior models because of the lack of moisture variance within the standardised reference values. The $\%Analyte_{DB}$ values were, however, important for comparison of different methods of drying and different drying conditions (Chapter 3).

Moisture content calibration (n = 150)

The distribution of MC of the sample set is shown in Figure 4.5. The MC calibration used 100 samples for the calibration set (range: 2.53% – 10.43%) and the remaining 50 for the independent validation set (range: 2.44% – 9.60%). For this particular calibration, pre-processing involved the use of a 2nd order polynomial Savitzky-Golay 1st derivative across 5 points.

The SEP for the MC calibration was determined as 0.24% (Table 4.3) and this value compared acceptably well with the SEL of 0.14% (Table 4.2). Furthermore a small bias of 0.01% and an excellent correlation coefficient (r) of 0.988 were also observed. Residual validation and calibration plots are given in Figure 4.6, showing the optimum number of PLS factors required for the MC calibration. The high correlation of this model is also apparent in the definite linear grouping of the validation scatter plot into low, middle and high MC value classes (Figure 4.7). The performance of this model compared adequately to FT-NIR moisture calibrations on ground wheat samples with SEP values of 0.15% (range: 8.55% to 12.70%; Manley *et al.*, 2002) and 0.19% (12.5% to 14.7%; Sorvaniemi *et al.*, 1993). Even though these values appear to be much lower than for the current study, the better performances of the wheat calibrations can partially be attributed to smaller MC ranges used.

After exclusion of particle variation by MSC, the largest remaining contributor to spectral variation was the moisture content. If a loadings line plot of the first few principle components (PC's) were to be plotted, it would produce spectra with strong absorption at *ca.* 1450 nm and 1930 nm (Figure 4.8). These wavelength regions correspond most strongly to the absorptions of water (or sample moisture), and although no single pure chemical or physical attribute can be linked to a particular PC (or PLS factor), it is evident that the high linearity of this model is affected by the moisture content of each sample. The relative contributions of each PLS factor to the explained variation are also large for the first few factors in the MC calibrations, with 97% explained after only 3 factors.

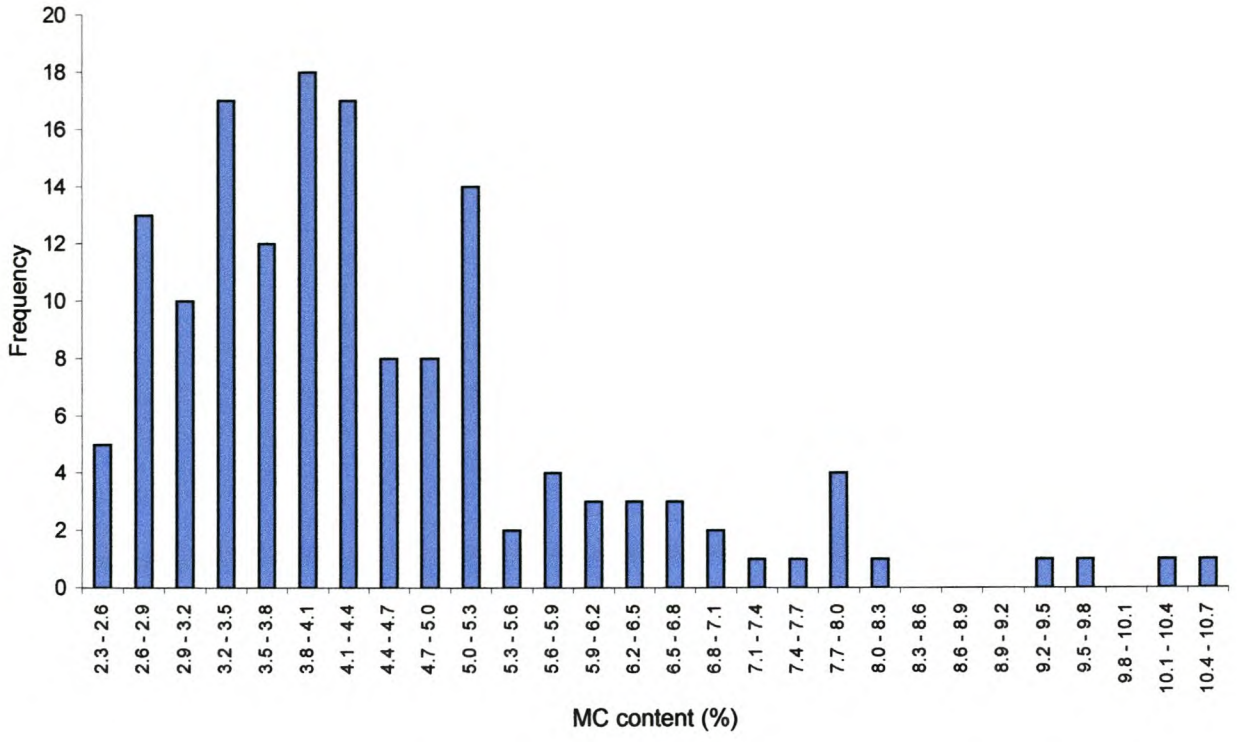


Figure 4.5 Histogram of the distribution of moisture content (MC) between samples of dried, ground Devil's Claw root (n = 150).

Table 4.3 Prediction results for the independently validated moisture content (MC) calibration from FT-NIR spectra of dried, ground Devil's Claw root.

	% Moisture (n = 150)	
	Calibration set	Validation set
Range	2.53 – 10.43	2.44 – 9.60
Mean	4.50	4.40
n	100	50
SEP	0.24	
Bias	0.01	
r	0.988	
PLS factors	4	

n = number of samples used in the calibration or validation sets, respectively.
SEP = standard error of prediction (independent validation set).
r = correlation coefficient.
PLS factors = number of factors used to build the calibration model.

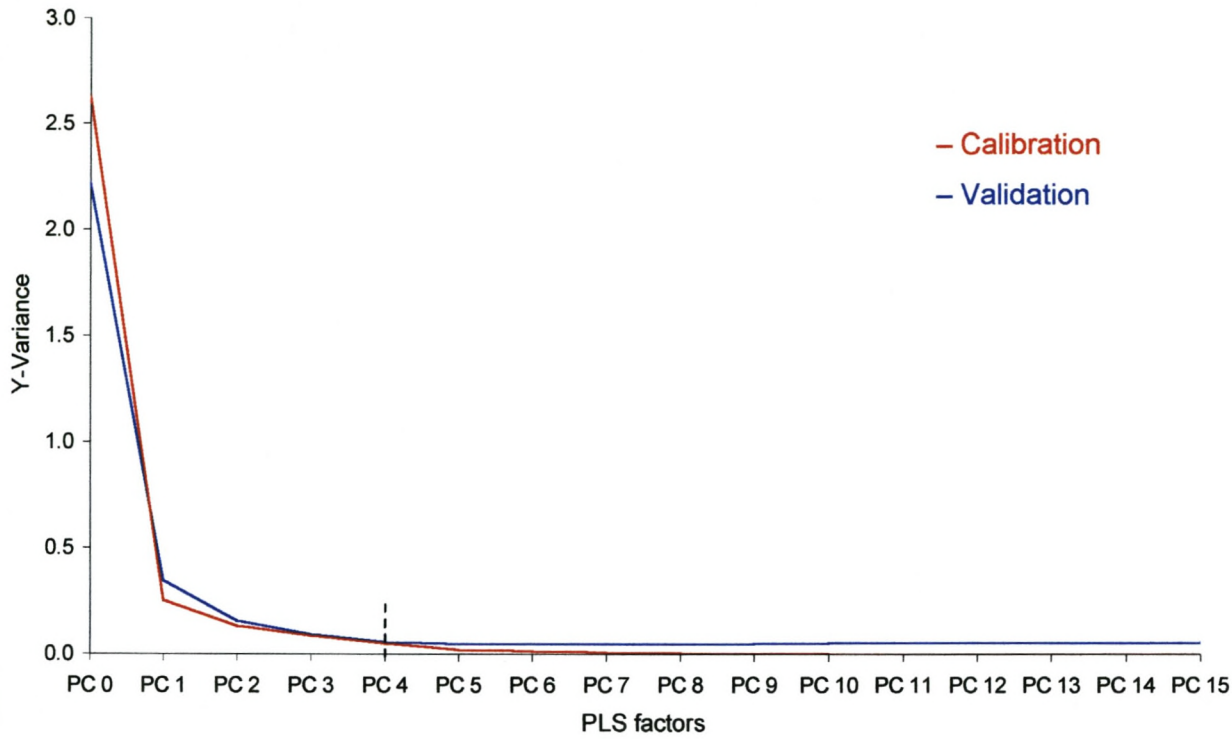


Figure 4.6 Residual validation and calibration plots for the FT-NIR moisture content model (4 factors used). Variances in the moisture content (y-axis) of dried, ground Devil's Claw samples are plotted against increasing partial least squares (PLS) factors.

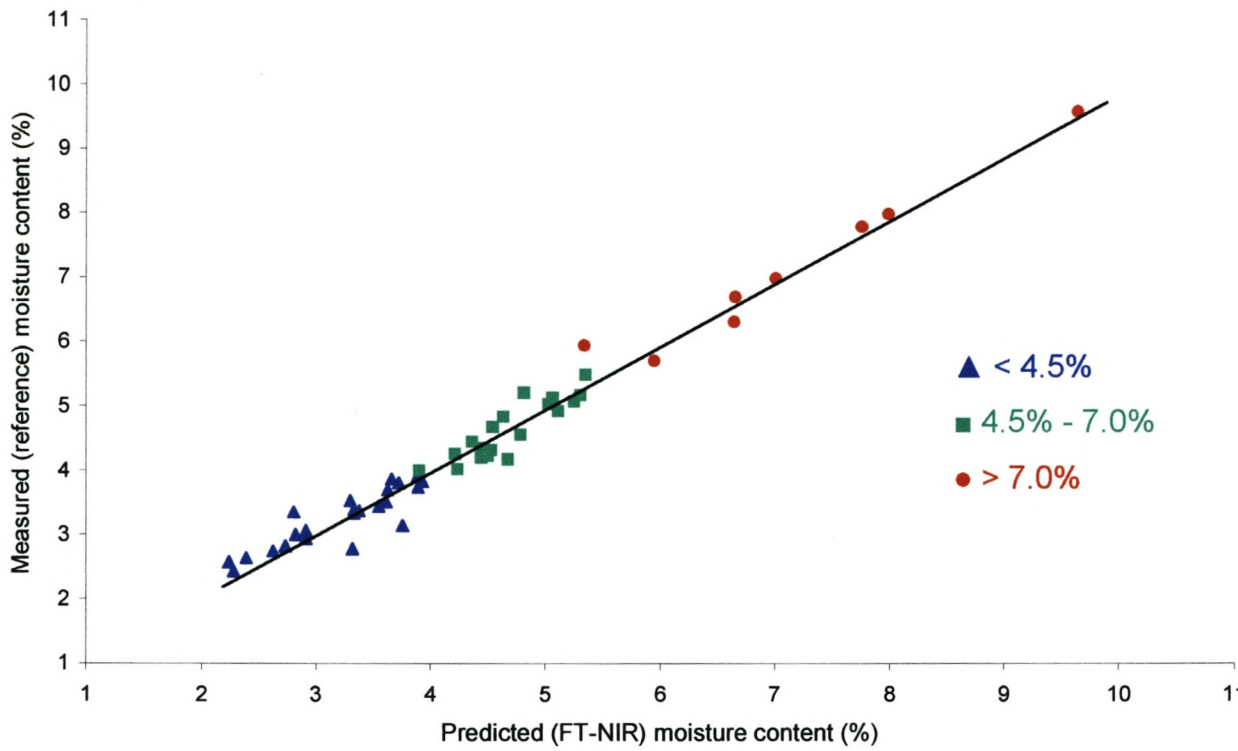


Figure 4.7 Scatter plot of the FT-NIR validation samples (n = 50) for the moisture content (MC) calibration model of dried, ground Devil's Claw root. Measured (reference) MC values are plotted against predicted (FT-NIRS) MC values. Three arbitrary MC ranges are indicated by the coloured groups, showing the high level of linearity in this model.

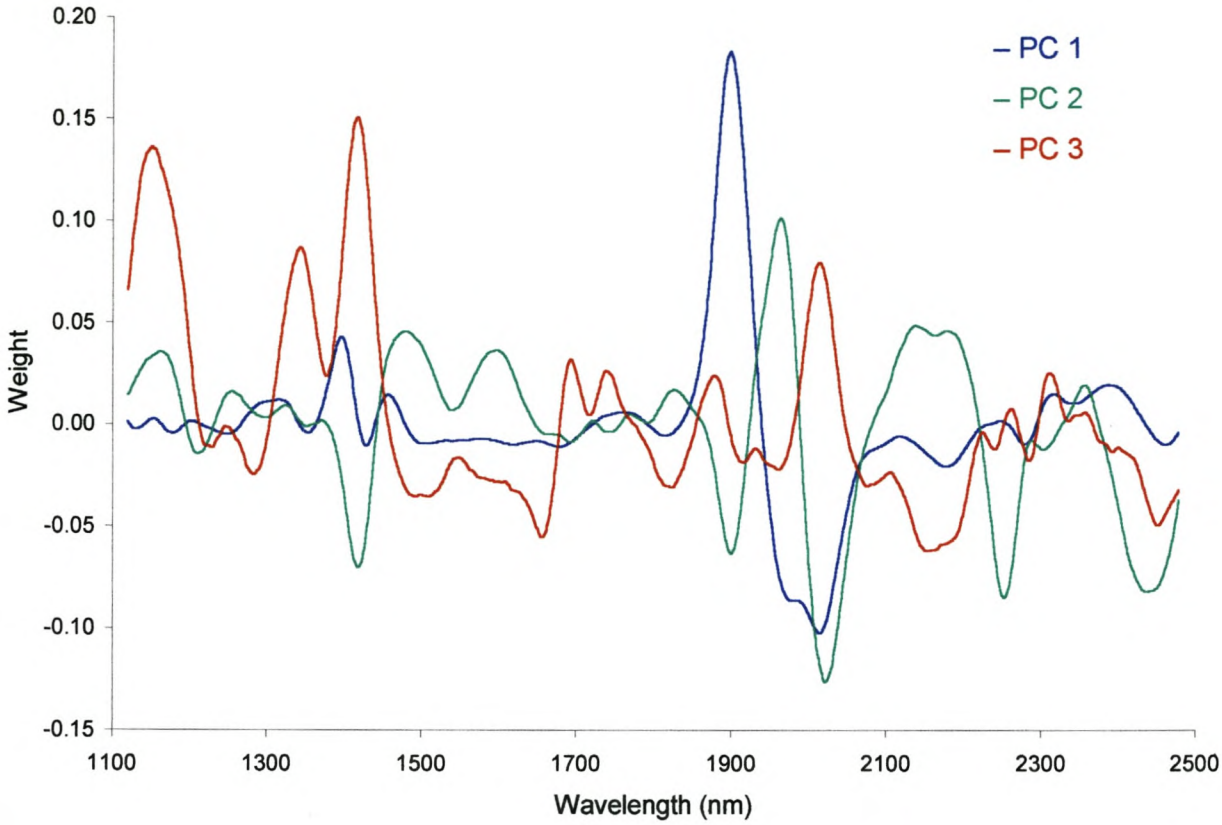


Figure 4.8 Line loadings plots of the first three FT-NIR principle components (PC 1, PC 2 and PC 3) from the moisture content (MC) calibration of dried, ground Devil's Claw root. The plot indicates the large influence of moisture variation (at *ca.* 1450 nm and 1930 nm) on the overall variation within the spectral data. In combination the three principle components explain 97% of the total MC variation.

The performance of the spectral data scanned on the NIRSystems 6500 instrument was poorer than the corresponding data from the Perkin-Elmer. Using the same reference values, the SEP of the 150-sample data set (after removal of one outlier) was 0.47% (Table 4.4). This value did not compare well with the calculated SEL of 0.14% (Table 4.2). A greater level of pre-processing was required, with MSC and second derivatives (Savitzky-Golay, segment size = 5) employed. A possible reason for the poorer performance could be the time required for the samples to reach Germany. Even though care was taken to ensure minimal moisture uptake, it is possible that the MC values differed from the reference values determined in South Africa before transport. This could have caused the lower correlation coefficient (r = 0.951) and predictive ability of the model. A total of 5 PLS factors was used for the NIRSystems MC model and the residual calibration and validation plot is given in Figure 4.9.

Harpagoside calibration (n = 150)

Although the differences between individual calibrations were often small, the best performance on the FT-NIR system for the HS model resulted from the raw (no MSC used) and first derivative (Savitzky-Golay, segment size = 11) treated data. The HS model on the NIRSystems 6500 performed best with the first derivative (Savitzky-Golay, segment size = 5) treated raw spectra.

The best FT-NIR HS model had an SEP of 0.236%, a bias of –0.048% and r of 0.641 (Table 4.5 and Figure 4.10). A total of 7 PLS factors were used and the residual calibration and validation plots are given in Figure 4.11. The SEP did not seem to compare well with the calculated SEL of 0.035% (Table 4.2), but the NIRS model was not expected to be as sensitive as the HPLC reference method. Prediction with this NIRS model should therefore not be used for quantitative purposes, but rather for screening to confirm the presence of HS in the root, and to classify the product into specified ranges. The main reason for the relatively poor prediction is that the overall $\%HS_{as\ is}$ range is very small, 0.693% - 2.244% (mean ± SD: 1.348% ± 0.299%), and even after calculating the $\%HS_{DB}$ content, the range remains low (0.721% - 2.436%; 1.413% ± 0.319%). The HS range also shows a near-Gaussian distribution (Figure 4.12) due to the randomness with which sample selection and preparation had occurred. This type of distribution will produce accurate estimations of HS close to the sample mean (*ca.* 1.4%), but less accurate results for samples near the extremes of the range (Osborne *et al.*, 1993). Although any future samples are also likely to have a Gaussian distribution, the robustness and accuracy of the HS model would best be increased if sample selection had created an even distribution.

The performance of the HS model improved when the NIRSystems 6500 spectra were used. After pre-processing with first derivatives (Savitsky-Golay, segment size = 13), the model

Table 4.4 Prediction results for the independently validated moisture (MC) and harpagoside (HS) contents calibrations from NIRSystems 6500 spectra of dried, ground Devil's Claw root.

	% Moisture (n = 150)	$\%HS_{as\ is}$ (n = 150)
SEP	0.47	0.134
Bias	0.04	0.012
r	0.951	0.901
PLS factors	5	11

SEP = standard error of prediction (independent validation set).
r = correlation coefficient.
PLS factors = number of factors used to build the calibration model.

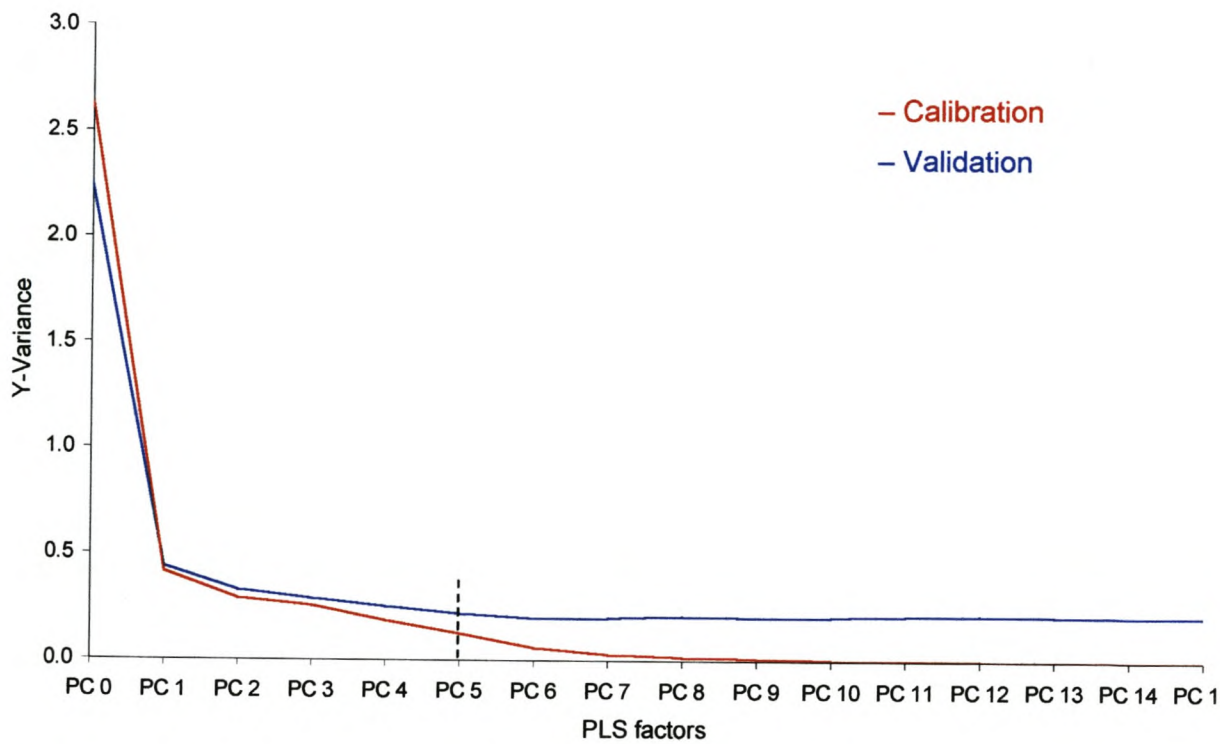


Figure 4.9 Residual validation and calibration plots for the NIRSystems 6500 moisture content model (5 factors used). Variances in the moisture content of dried, ground Devil's Claw samples are plotted against increasing partial least squares (PLS) factors.

Table 4.5 Prediction results for the independently validated harpagoside (HS) content calibration from FT-NIR spectra of dried, ground Devil's Claw root.

	$\%HS_{as\ is}$ (n = 150)	
	Calibration set	Validation set
Range	0.803 – 2.244	0.693 – 2.129
Mean	1.354	1.337
n	100	50
SEP	0.236	
Bias	-0.048	
r	0.641	
PLS factors	7	

n = number of samples used in the calibration or validation sets, respectively.
SEP = standard error of prediction (independent validation set).
r = correlation coefficient.
PLS factors = number of factors used to build the calibration model.

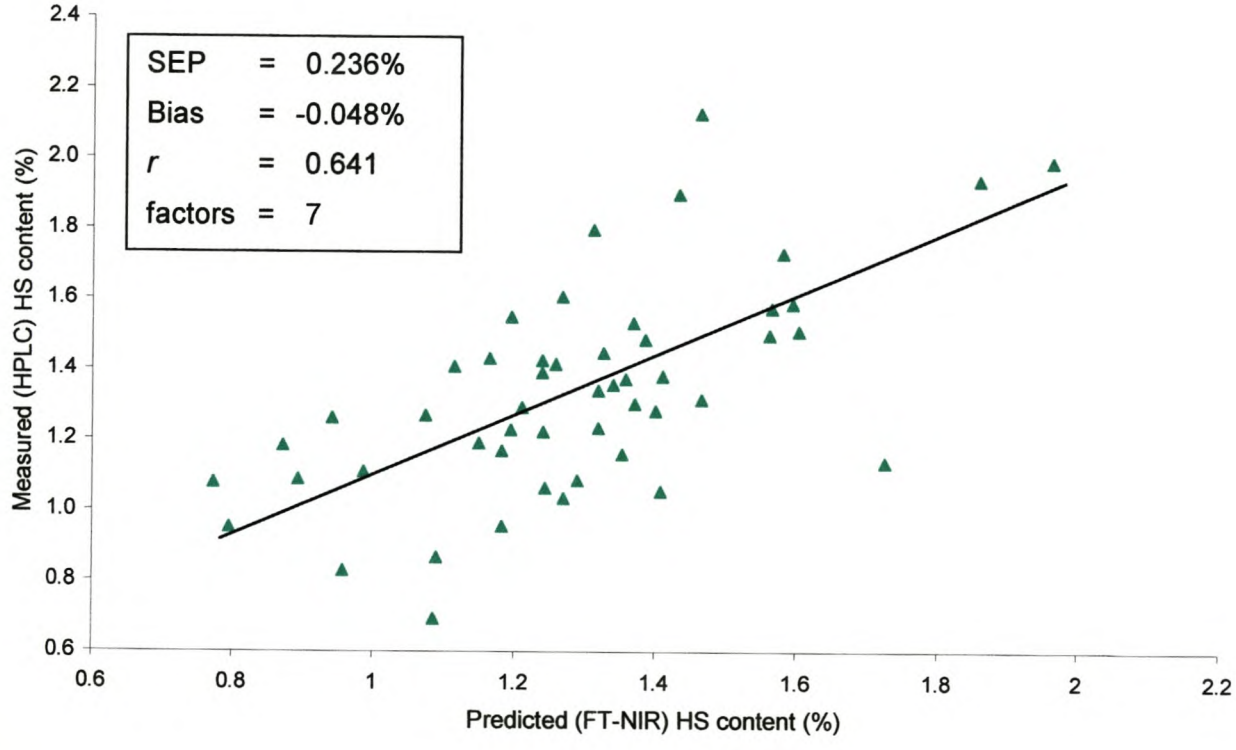


Figure 4.10 Scatter plot for the validation samples (n = 50) for the FT-NIR harpagoside (HS) calibration model. Reference HS values (determined by HPLC) are plotted against predicted (Perkin-Elmer) HS values.

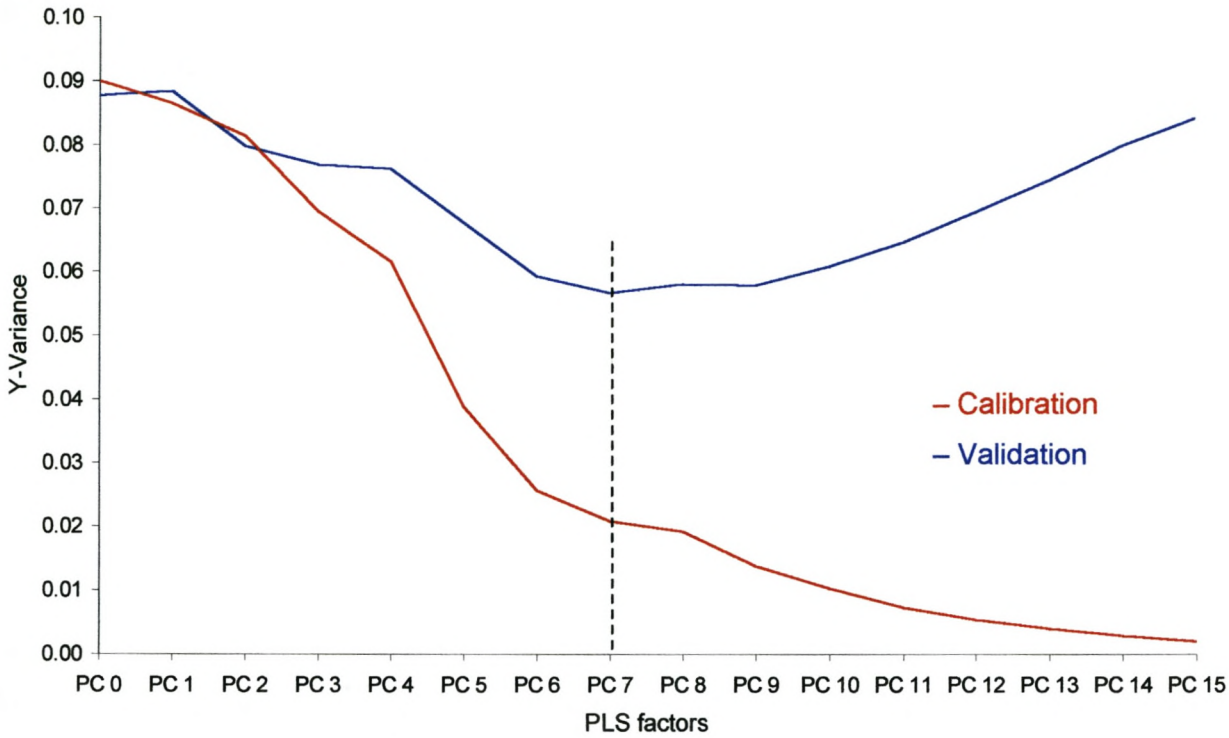


Figure 4.11 Residual validation and calibration variance plot for the FT-NIR harpagoside (HS) content model (7 factors used). Variances in the HS content of dried, ground Devil's Claw samples are plotted against increasing partial least squares (PLS) factors.

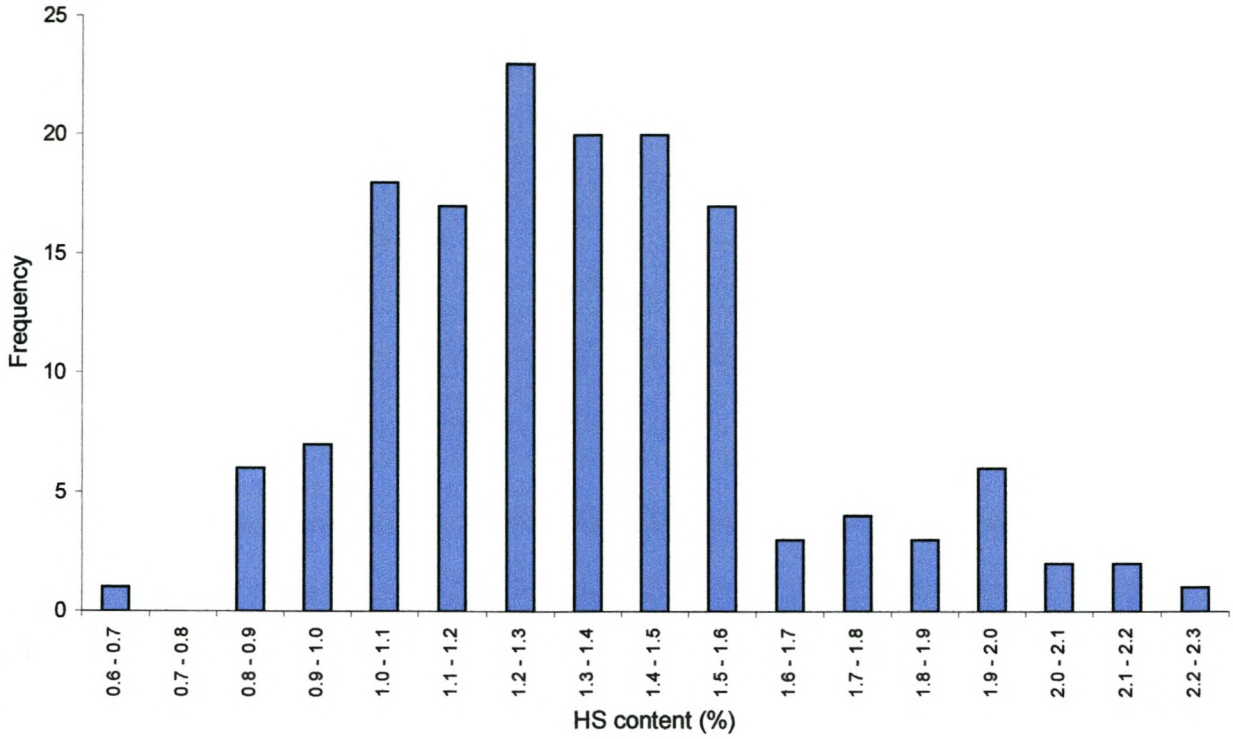


Figure 4.12 Histogram of the distribution of harpagoside (HS) content between samples of dried, ground Devil's Claw root ($n = 150$).

had an SEP of 0.134% (Table 4.4) that compared much better to the SEL of 0.035% (Table 4.2). A much higher correlation coefficient (r = 0.901) was also observed (Figure 4.13). The model required 11 PLS factors and the residual HS variances for the calibration and validation models are indicated in Figure 4.14. Although fewer PLS factors (e.g. 3) would have given a more robust HS model with reduced accuracy.

When comparing the spectra from the two instruments, the most evident difference is the additional peak at *ca.* 2200 nm (Figure 4.15). According to Osborne *et al.* (1993), this area of the NIR spectrum correlates to the –CH and –C=O stretching vibrations in a –CHO structure. Additionally, bands at 2190 nm also correlate with the stretching vibrations of –C=C– bonds. Part of the trans-cinnamoyl and ρ-coumaroyl moieties, in HS and 8ρCHG respectively, have a –HC=CH–C=O structure. The better definition of the peak in this area of the Foss NIRSystems 6500 spectra highlights variances in the iridoid content and leads, subsequently, to improved NIRS models.

8-ρ-Coumaroyl harpagide calibration (n = 42)

The effect on 8ρCHG calibrations of the exclusion of the *H. zeyheri* samples can clearly be seen by the change in predictive ability of the full cross-validated FT-NIR models as illustrated in Table 4.6. The SECV of the FT-NIR model improved from 0.092% (Figure 4.16) to 0.035% with the exclusion of the two *H. zeyheri* samples.

As before, the reference method (SEL = 0.007%) far outperformed the NIRS models in terms of sensitivity and accuracy, but NIRS cannot be expected to compete quantitatively with HPLC in cases where the analyte levels are this low.

Although the SECV values did not approach the SEL values very closely, NIRS calibrations with relatively high correlation coefficients (r) were developed. The better r for the 8ρCHG models, when compared to the equivalent HS models, can be attributed to the relatively larger concentration range (0.072% - 0.301% for %8ρCHG$_{DB}$, excluding *H. zeyheri*), even though the compound occurs in much lower concentrations in the root. The potential exists to utilise such models for 8ρCHG screening, especially if the screening was aimed at classifying samples for species distinction. Support for this may be found in the PCA plot where the two *H. zeyheri* samples are clearly separated from the *H. procumbens* group (Figure 4.17). A greater number of authentic *H. zeyheri* samples (verified by HPLC or TLC) is, however, required to confirm the feasibility of using PCA analysis as a species classification tool.

Models using NIRSystems 6500 spectra improved slightly on the FT-NIR models. Pre-processing of these spectra involved MSC and 1st derivates, and resulted in an SECV of 0.058%

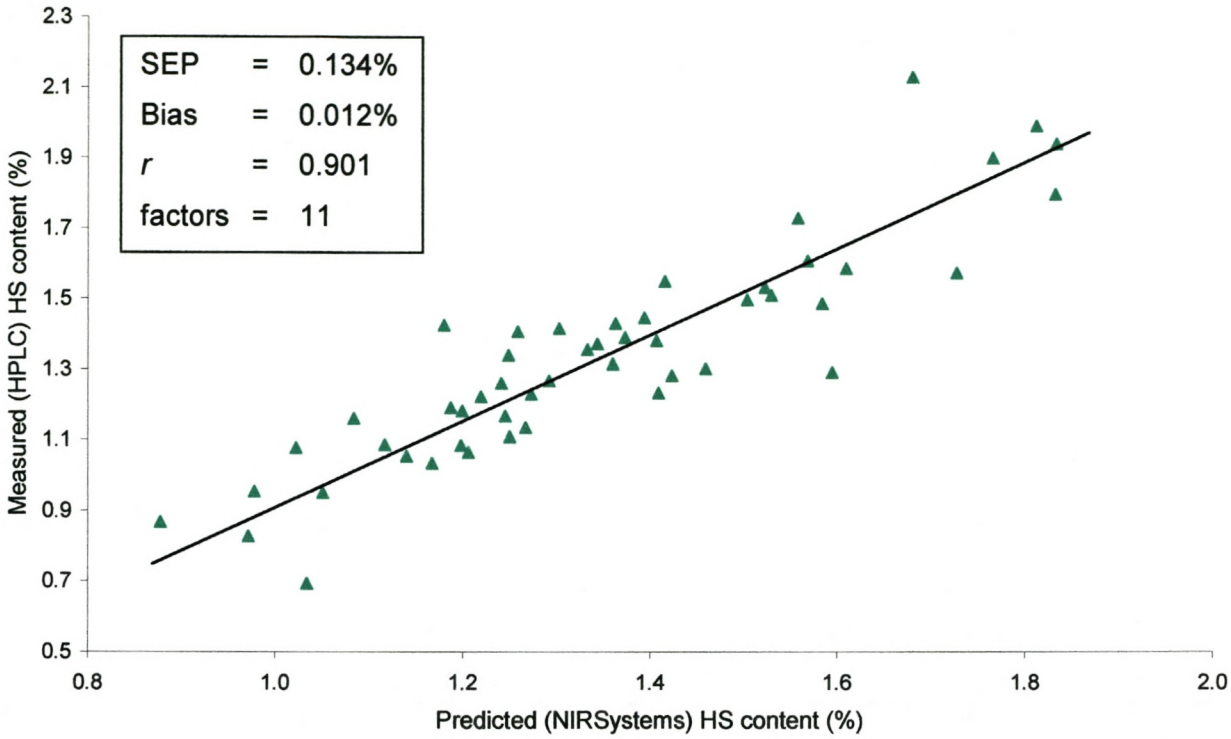


Figure 4.13 Scatter plot of the validation samples (n = 50) for the NIRSystems 6500 harpagoside (HS) calibration model. Reference HS values (determined by HPLC) are plotted against predicted (Foss) HS values.

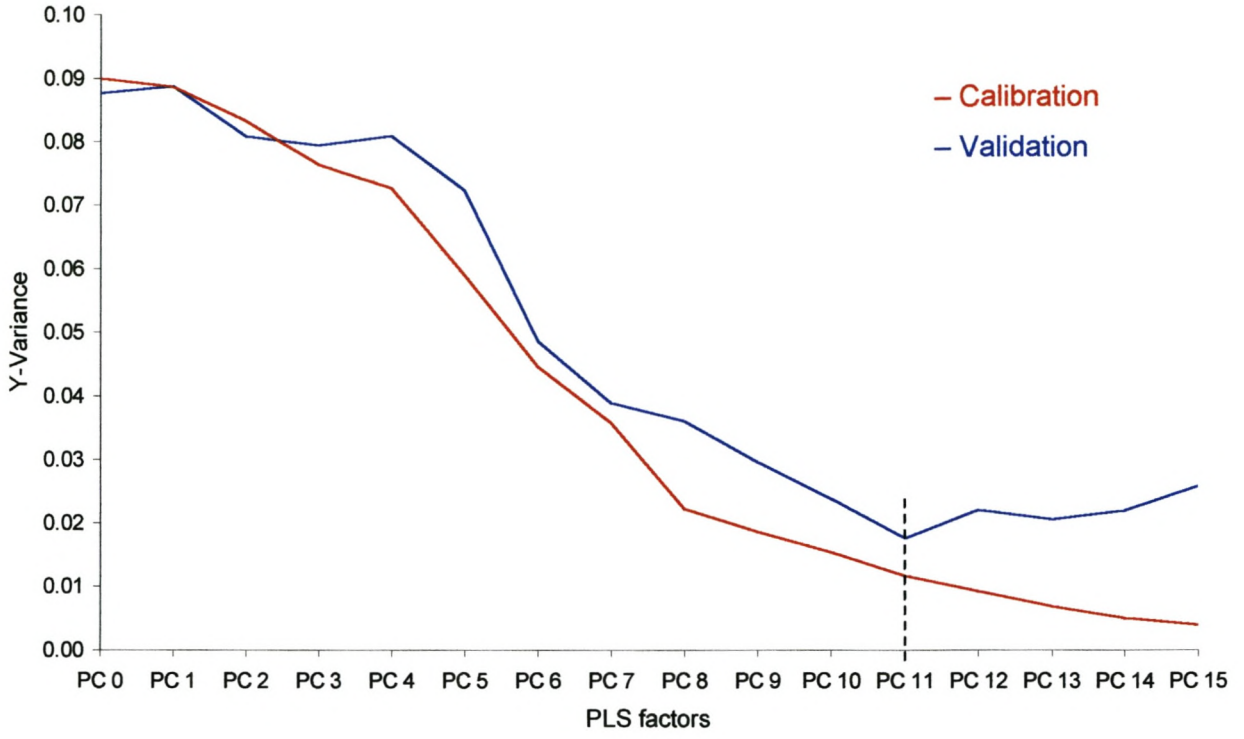


Figure 4.14 Residual validation and calibration variance plot for the NIRSystems harpagoside (HS) content model (11 factors used). Variances in the HS content of dried, ground Devil's Claw samples are plotted against increasing partial least squares (PLS) factors.

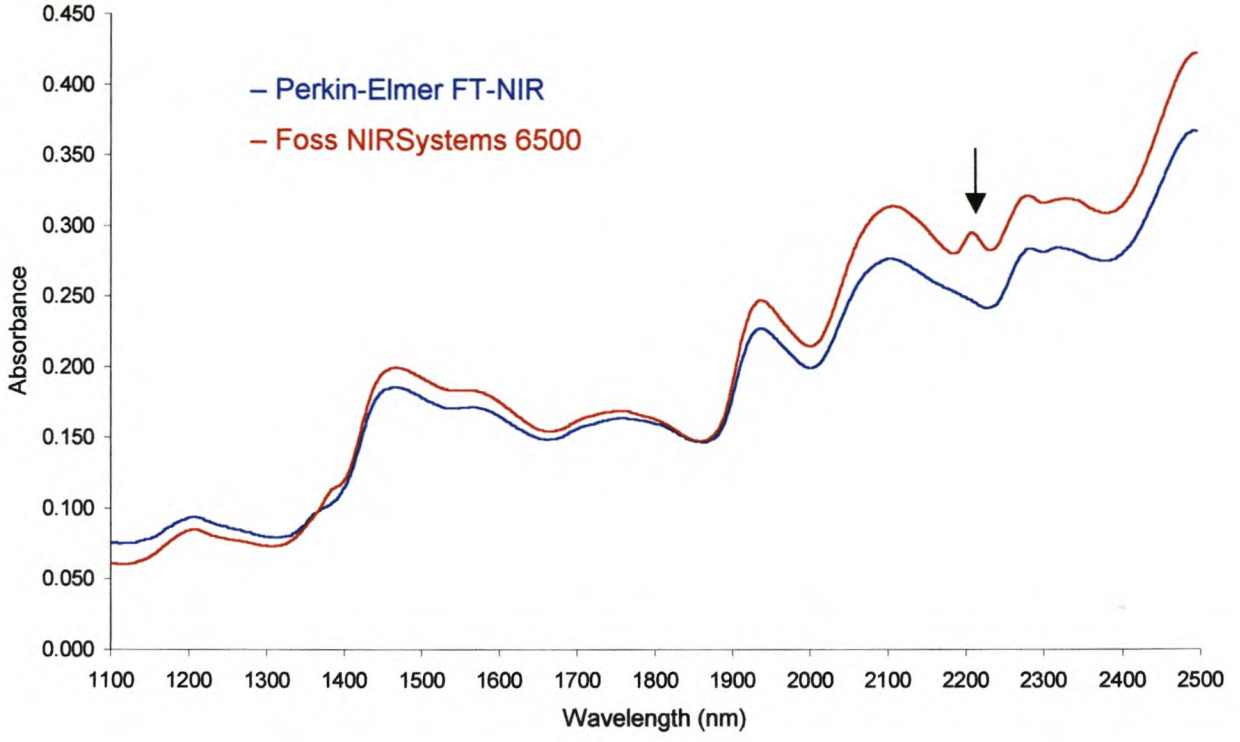


Figure 4.15 Comparison of the spectra of a single Devil's Claw sample, scanned by the Perkin-Elmer FT-NIR IdentiCheck and Foss NIRSystems 6500 instruments. A slight rotational difference, probably due to sample presentation, is evident. An additional well-defined peak is evident in the Foss spectrum at 2190 nm (indicated by the arrow).

Table 4.6 Summary of the predictive performance of the full-cross validated 8-ρ-coumaroyl harpagide (8ρCHG) content calibrations from FT-NIR spectra of dried, ground Devil's Claw root.

	%8ρCHG$_{as\ is}$ including *H. zeyheri*	**%8ρCHG$_{as\ is}$ excluding *H. zeyheri***
	Cross-validation set	Cross-validation set
Range	0.069 – 0.957	0.069 – 0.290
Mean	0.175	0.136
n	42	40
SECV	0.092	0.035
Bias	-0.005	0.000
r	0.873	0.846
PLS factors	6	6

n = number of samples used in full cross-validation.
SECV = standard error of cross validation (full-cross validation).
r = correlation coefficient.
PLS factors = number of factors used to build the calibration model.

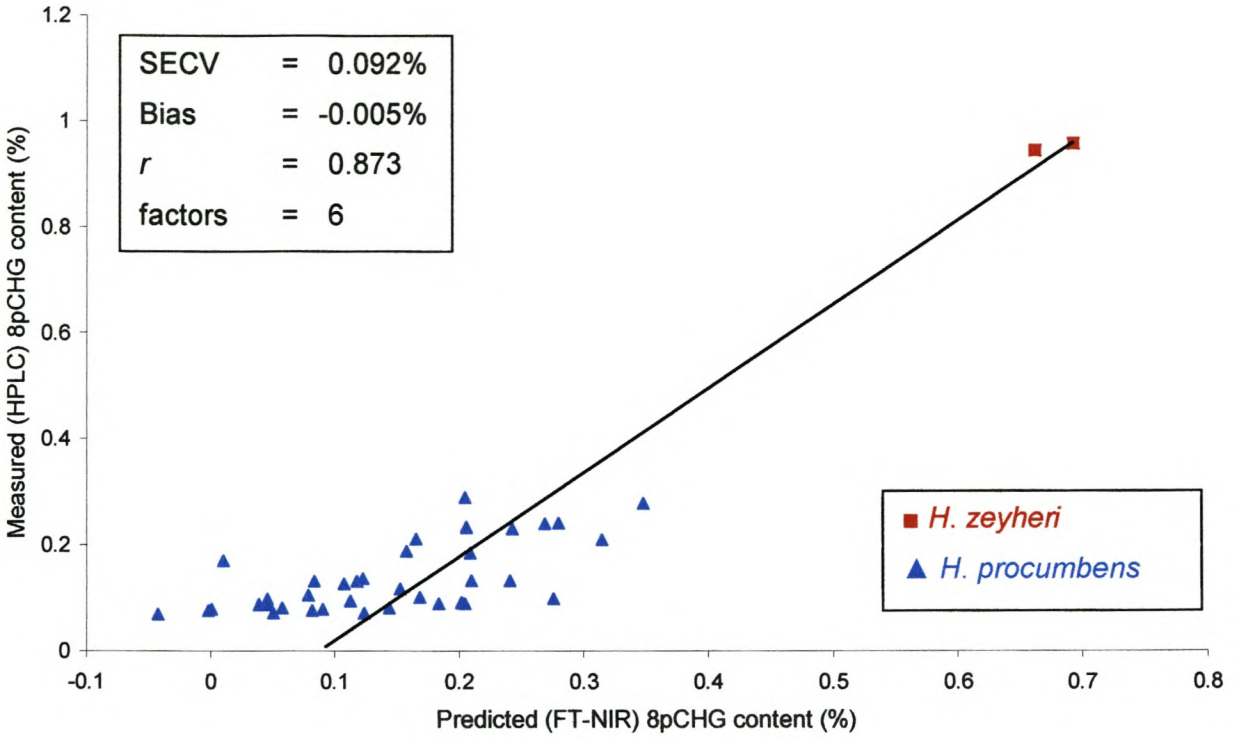


Figure 4.16 Scatter plot of the full cross-validated samples (n = 42) for the FT-NIR 8-ρ-coumaroyl harpagide (8ρCHG) calibration model. Reference 8ρCHG values (determined by HPLC) are plotted against predicted (FT-NIRS) 8ρCHG values with *Harpagophytum zeyheri* samples grouped away due to high 8ρCHG values.

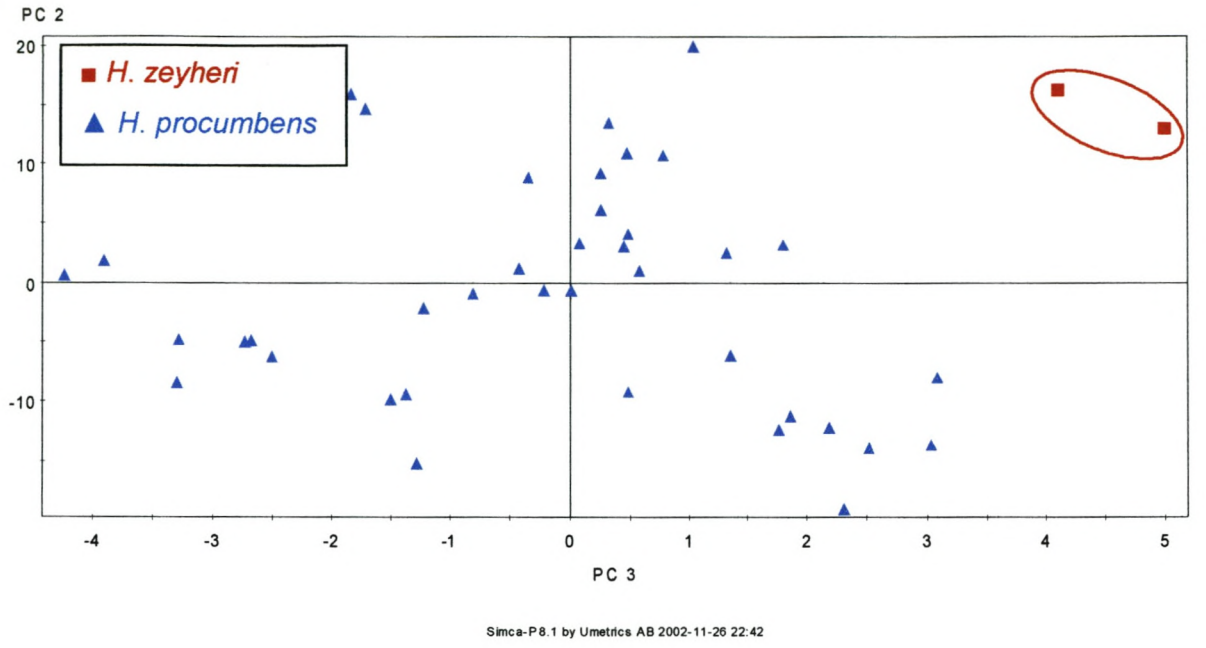


Figure 4.17 PCA plot from FT-NIR spectra of 42 dried, ground Devil's Claw samples indicating the possible classification of *Harpagophytum procumbens* and *H. zeyheri*. The second and third principle components are plotted against one another, since the first PC explains mostly particle size variation in these unprocessed spectra.

and an *r* of 0.952 (Table 4.7). As was the case with FT-NIR models, a further improvement was observed upon removal of the *H. zeyheri* samples, with the SECV = 0.028% and an *r* of 0.906 (Figure 4.18). Although these performances were better than for the FT-NIR data, a greater number of PLS factors were required (12) to achieve it. Figure 4.19 shows the residual 8ρCHG variance for the calibration and validation sets. Although over-fitting remains a danger, the better performance may be linked to the additional peak observed in the NIRSystems spectra.

Harpagoside:8-ρ-Coumaroyl harpagide ratio calibration (*n* = 42)

The ratio between HS and 8ρCHG for each duplicate sample was calculated from the original integration values (μV.s) and used for development of ratio calibrations. It can be argued that the $\%Analyte_{DB}$ reference values would provide more accurate ratios, but the samples were originally scanned on a wet basis and as such, reference values with moisture variation included should be used. The main results (unadjusted data with *H. zeyheri* included) are shown in Table 4.8 and Figure 4.20.

The upper part of the ratio range (17.13 to 34.48) for confirmed *H. procumbens* samples corresponded reasonably well with the ranges found by Baghdikian *et al.* (1997) and Eich *et al.* (1998). The minimum value (5.62) was, however, much lower than the published values of 20 (Baghdikian *et al.*, 1997) and 17 (Eich *et al.*, 1998). Although it had been suggested by these authors that the ratio be used to distinguish *Harpagophytum* species, this study found that the two *H. zeyheri* samples (1.84) were not separated well enough (Figure 4.20) from the overall *H. procumbens* range (5.62 to 34.48; average: 15.83) to validate species distinction based solely on this parameter.

Unfortunately, since only two confirmed *H. zeyheri* samples could be included in any of the data sets, it was difficult to estimate whether these two would form the upper, middle or lower end for that particular range. If, as reported (Baghdikian *et al.*, 1997; Eich *et al.*, 1998), the *H. zeyheri* ratios ranged from *ca.* 1 to 2, it might still be possible to use this ratio for species distinction, however, the minimum limit for true *H. procumbens* samples would have to be lowered from the suggested ratio of 17 to 5 or even less.

Eich *et al.* (1998) suggested that the actual %8ρCHG value rather than HS:8ρCHG ratio should be used to distinguish the species. The results in Table 4.6 certainly indicate a large difference between the 8ρCHG value for confirmed *H. zeyheri* (*ca.* 0.9) and the maximum value for *H. procumbens* (*ca.* 0.3). Based on the results from the present study, it was concluded that the greater separation of the species would most probably be found through direct comparison of only the 8ρCHG concentration. Determination of the HS values would then serve to classify the

Table 4.7 Summary of the predictive performance of the full-cross validated 8-ρ-coumaroyl harpagide (8ρCHG) content calibrations from NIRSystems 6500 spectra of dried, ground Devil's Claw root.

	%8ρCHG$_{as\ is}$ (42 data set) including *H. zeyheri*	**%8ρCHG$_{as\ is}$ (42 data set) excluding *H. zeyheri***
n	42	40
SECV	0.058	0.028
Bias	-0.005	-0.000
r	0.952	0.906
PLS factors	12	12

n = number of samples used in full cross-validation.
SECV = standard error of cross validation (full-cross validation).
r = correlation coefficient.
PLS factors = number of factors used to build the calibration model.

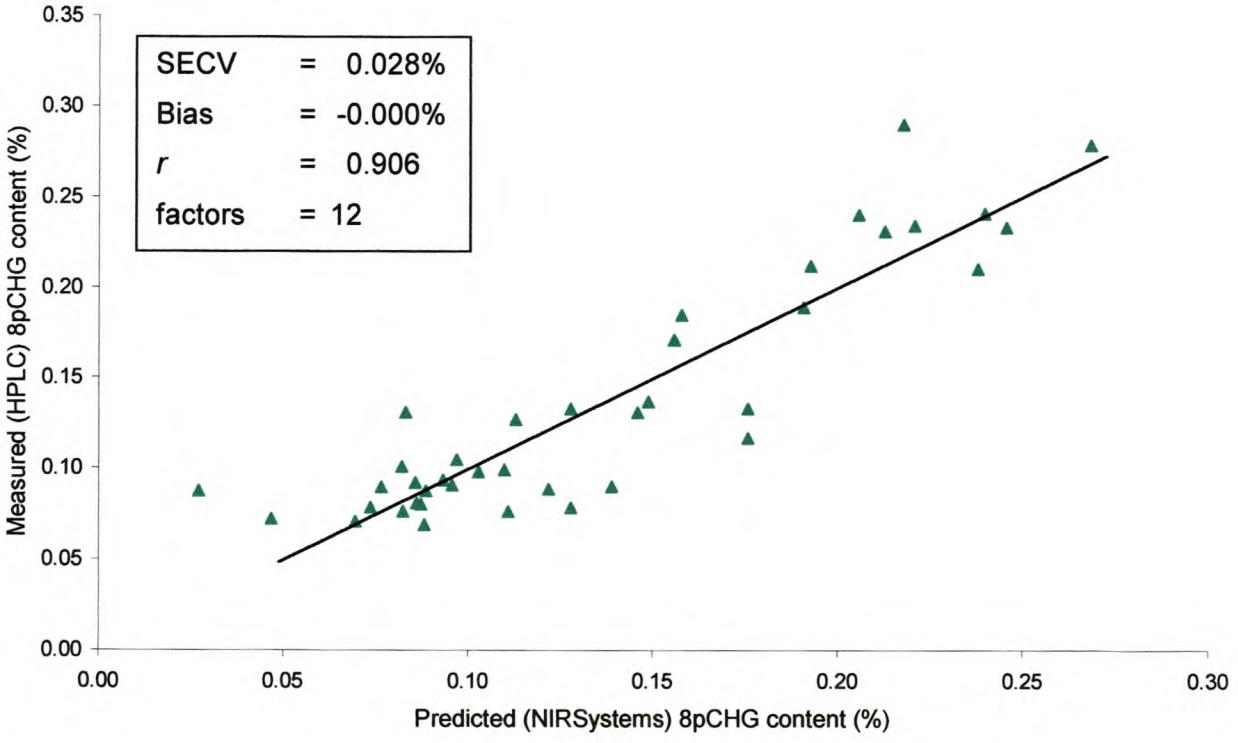


Figure 4.18 Scatter plot of the full cross-validated samples (*n* = 40) for the NIRSystems 6500 8-ρ-coumaroyl harpagide (8ρCHG) calibration model. Reference 8ρCHG values (determined by HPLC) are plotted against predicted (Foss) 8ρCHG values.

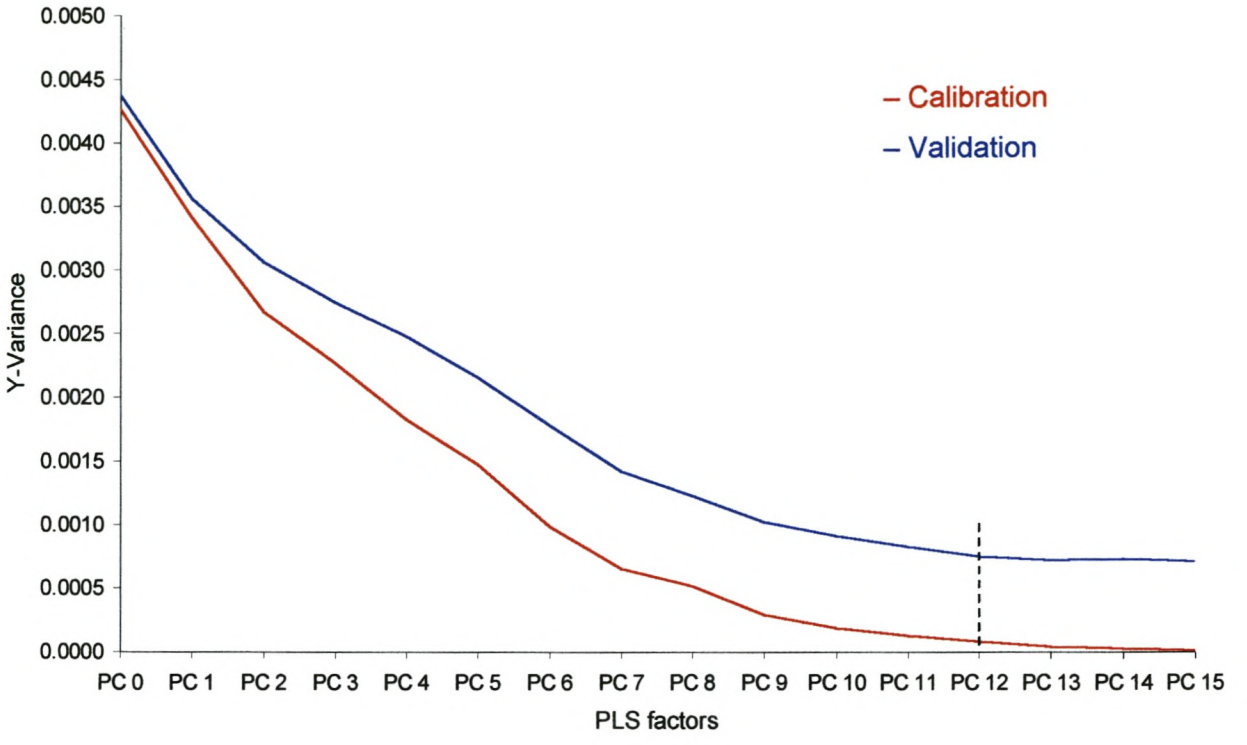


Figure 4.19 Residual validation and calibration variance plots for the NIRSystems 8-ρ-coumaroyl harpagide (8ρCHG) content model (12 factors used). Variances in the 8ρCHG content of dried, ground Devil's Claw samples are plotted against increasing partial least squares (PLS) factors.

Table 4.8 Summary of the predictive performance of the full-cross validated harpagoside:8-ρ-coumaroyl harpagide ratio calibrations from FT-NIR spectra of dried, ground Devil's Claw root.

	HS:8pCHG ratio including *H. zeyheri*	**HS:8pCHG ratio (42 data set) excluding *H. zeyheri***
	Cross-validation set	Cross-Validation set
Range	1.84 – 34.48	5.62 – 34.48
Mean	15.17	15.83
n	42	40
SECV	3.86	4.10
Bias	0.17	0.10
r	0.873	0.839
PLS factors	10	9

n = number of samples used in full cross-validation.
SECV = standard error of cross validation (full-cross validation).
r = correlation coefficient.
PLS factors = number of factors used to build the calibration model.

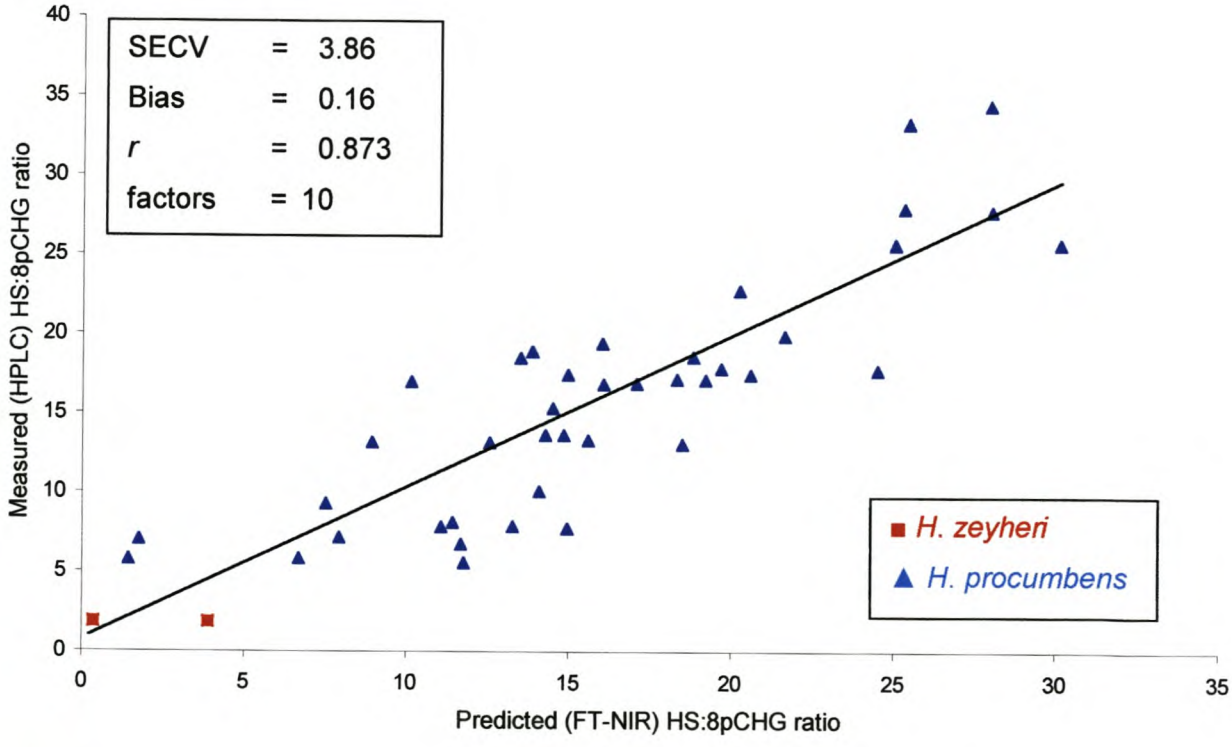


Figure 4.20 Scatter plot of the full cross-validated samples (*n* = 42) for the FT-NIR harpagoside:8-ρ-coumaroyl harpagide ratio calibration model. The ratios between the analyte values are plotted against predicted (Perkin-Elmer) ratios.

medicinal potency or compound availability within the roots.

The performance of the FT-NIR ratio model, was compared to the corresponding NIRSystems 6500 model. The results are summarised in Table 4.9. As with the individual analyte calibrations, the NIRSystems spectra seemed to correlate better with the ratio reference values. The SECV for the 42-sample calibrations improved from FT-NIR value of 3.86 to 2.87 when using these spectra. This improvement may be mostly ascribed to the difference in performance of the HS calibrations. The additional peak at *ca.* 2200 nm (Figure 4.15) may add to the explanation of both HS and 8ρCHG variation within the NIRSystems 6500 spectra.

8-ρ-Coumaroyl harpagide calibration (n = 150; adjusted)

To facilitate the development of a more robust 8ρCHG calibration, a 150-sample data set was constructed by combining the 42 samples, with accurate 8ρCHG values, and 108 samples with adjusted 8ρCHG values. The adjustment of 8ρCHG values was calculated from the relative response of 8ρCHG standards to detection at 278 nm and 312 nm.

The influence of the *H. zeyheri* samples on the NIRS models with accurate 8ρCHG values was clearly illustrated (Tables 4.6 and 4.7). The adjusted 150-sample data set indicated similar effects, although the predictive ability had deteriorated. *Harpagophytum procumbens* samples showed a more even distribution of 8ρCHG content than for HS content, with *H. zeyheri* clearly separated (Figure 4.21). An even distribution facilitates the development of accurate NIR models across a larger range of the estimated analyte (Osborne *et al.*, 1993).

The best FT-NIRS model, including the *H. zeyheri* samples, had an SEP of 0.095%, a bias of –0.006% and r of 0.708. These values improved to an SEP of 0.051% upon the removal of the *H. zeyheri* samples. A further improvement was found once two outliers had been removed, with a final SEP of 0.048% (Figure 4.22).

The performance of the adjusted 8ρCHG reference values for the NIRSystems 6500 spectra, including *H. zeyheri* samples, was very similar to the Perkin-Elmer data. After MSC and 1st derivative treatment, the calibration had an SEP of 0.084% and an r of 0.776. The removal of the *H. zeyheri* samples did, however, not improve the predictive ability as much as the FT-NIRS calibration had done. The SEP only improved to 0.054% compared to the FT-NIR SEP of 0.048%. Both of the final models only required 5 PLS factors, making them more robust (Table 4.10).

Once again, PCA analysis showed separation between *H. procumbens* and *H. zeyheri*

Table 4.9 Summary of the predictive performance of the full-cross validated harpagoside:8-ρ-coumaroyl harpagide ratio calibrations from NIRSystems 6500 spectra of dried, ground Devil's Claw root.

	%8ρCHG$_{as\ is}$ including *H. zeyheri*	**%8ρCHG$_{as\ is}$ excluding *H. zeyheri***
n	42	40
SECV	2.87	3.10
Bias	0.06	-0.02
r	0.933	0.912
PLS factors	11	11

n = number of samples used in full cross-validation.
SECV = standard error of cross validation (full-cross validation).
r = correlation coefficient.
PLS factors = number of factors used to build the calibration model.

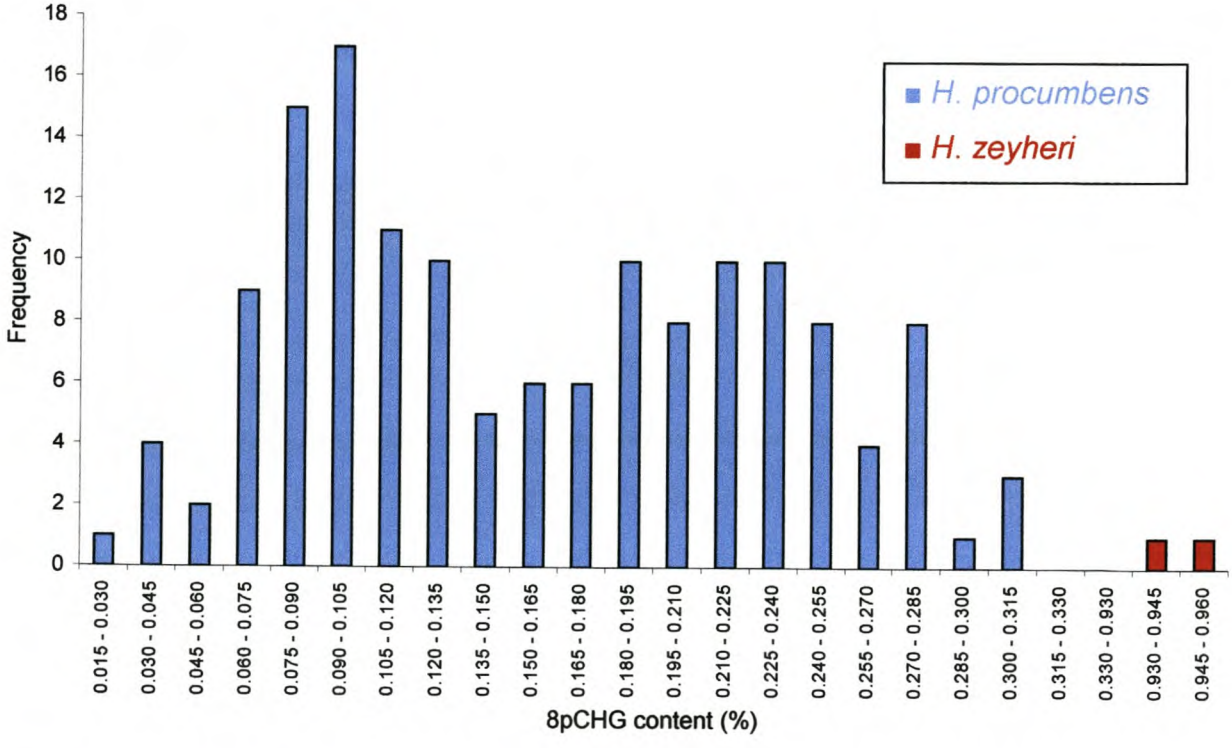


Figure 4.21 Histogram of the distribution of 8-ρ-coumaroyl harpagide (8ρCHG) content between samples of dried, ground Devil's Claw root (n = 150; adjusted). The two *Harpagophytum zeyheri* samples are clearly separated at the maximum extreme of the range.

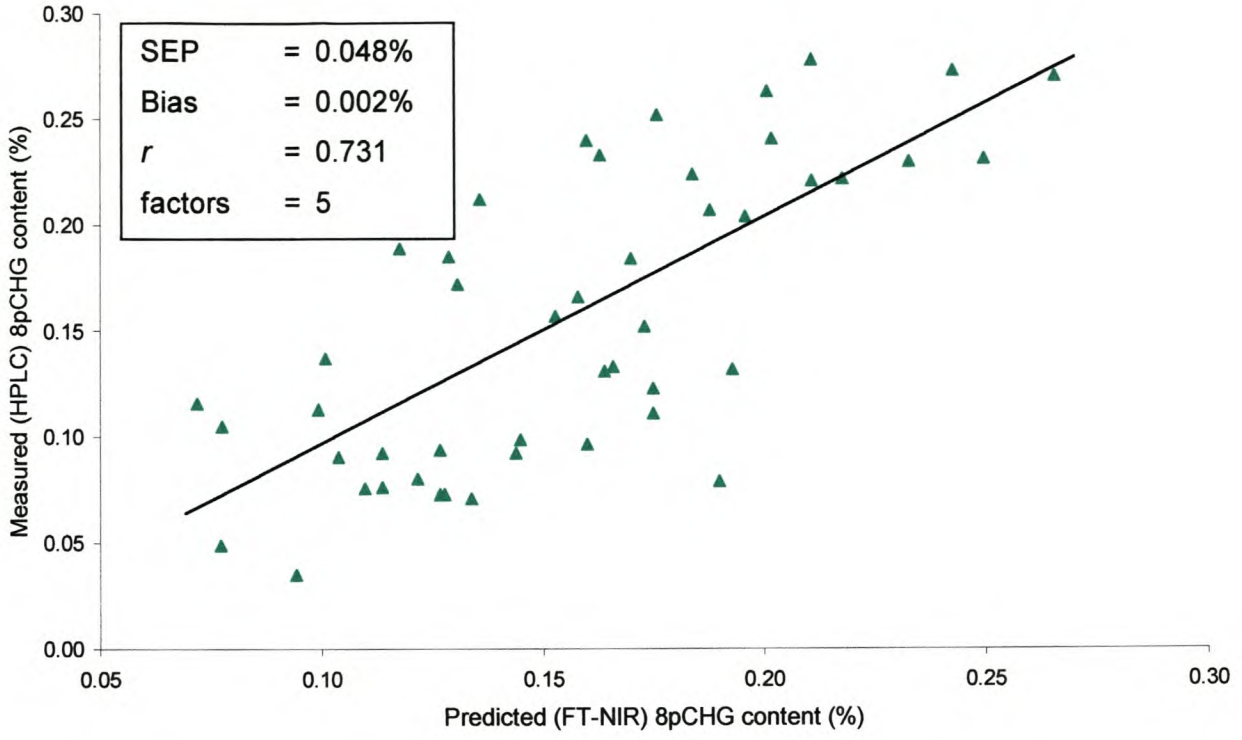


Figure 4.22 Scatter plot of the validation samples (n = 47; adjusted) for the FT-NIR 8-ρ-coumaroyl harpagide (8ρCHG) calibration model. Reference 8ρCHG values (adjusted) are plotted against predicted (Perkin-Elmer) 8ρCHG values.

Table 4.10 Summary of the predictive performance for 8-ρ-coumaroyl harpagide (8ρCHG) content using the FT-NIR and NIRSystems spectra of dried, ground Devil's Claw root, respectively. The *Harpagophytum zeyheri* samples have been removed from both calibrations, as well as selected outliers.

	%8ρCHG$_{\text{as is}}$ Perkin-Elmer FT-NIR	**%8ρCHG$_{\text{as is}}$ Foss NIRSystems 6500**
n	146	148
SEP	0.048	0.054
Bias	0.002	-0.001
r	0.731	0.657
PLS factors	5	5

n = total number of samples used (calibration and validation sets).
SEP = standard error of prediction (independent validation set).
r = correlation coefficient.
PLS factors = number of factors used to build the calibration model.

samples (Figure 4.23). Similar results were obtained from the NIRSystems 6500 spectra (data not shown). It is important to note that the spectral data was not adjusted and, consequently, the results from this PCA analysis lend further support to species classification by PCA.

Following the performances of both the unadjusted (n = 42) and the adjusted (n = 150) data sets, it is recommended that species distinction be based upon 8ρCHG values. Even with a relatively high SEP of 0.095% for the FT-NIR model, the difference in 8ρCHG concentrations between *H. procumbens* and *H. zeyheri* (*ca.* 0.5%) still shows that the model would good enough to distinguishing between the two species. The robustness of such a model does, however, need to be improved by inclusion of a large number of authenticated *H. zeyheri* samples.

Harpagoside:8-ρ-coumaroyl harpagide ratio calibration (n = 150; adjusted)

Generally, the ratio calibrations suffered most from the adjustment of 8ρCHG values. The MSC and 1st derivative (Savitsky-Golay, segment size = 11) data produced a model with an SEP of 6.55, bias of -0.392 and r of 0.584 for the FT-NIR spectra.

The removal of confirmed outliers once again improved the model's performance for this data set. One outlier in particular had a marked effect on the calibration. At a ratio of 51.49, this sample defined the maximum value of the range. The nearest sample had a value of 34.48 and the average ratio only changed by 0.21 when 51.49 was removed. The distribution of samples based on HS:8ρCHG ratio is shown in Figure 4.24. It was accepted that since a ratio does not define or reflect the natural variation of a single component, and since the values for this sample had to be adjusted, the sample could be classified as an outlier. After removal of this and two further outliers, the SEP improved to 5.66 with an r of 0.603 (Table 4.11). The comparative NIRSystems model (2nd derivative, segment size = 5) achieved similar results with an SEP of 5.30 and an r of 0.610.

Comparative drying studies

In addition to the study of the effect that different drying methods and drying temperatures had on HS content in Devil's Claw root (Chapter 3), the NIR spectra (n = 51) were also analysed by PCA. The PCA plot for the drying method study is given in Figure 4.25 and the temperature study in Figure 4.26.

It is clear from Figure 4.25 that a definite grouping between the three drying methods (sun, tunnel and freeze-drying) can be seen in the PCA plot. This grouping is, however, most likely due to the significant MC differences between the three drying methods (data not shown). NIRS

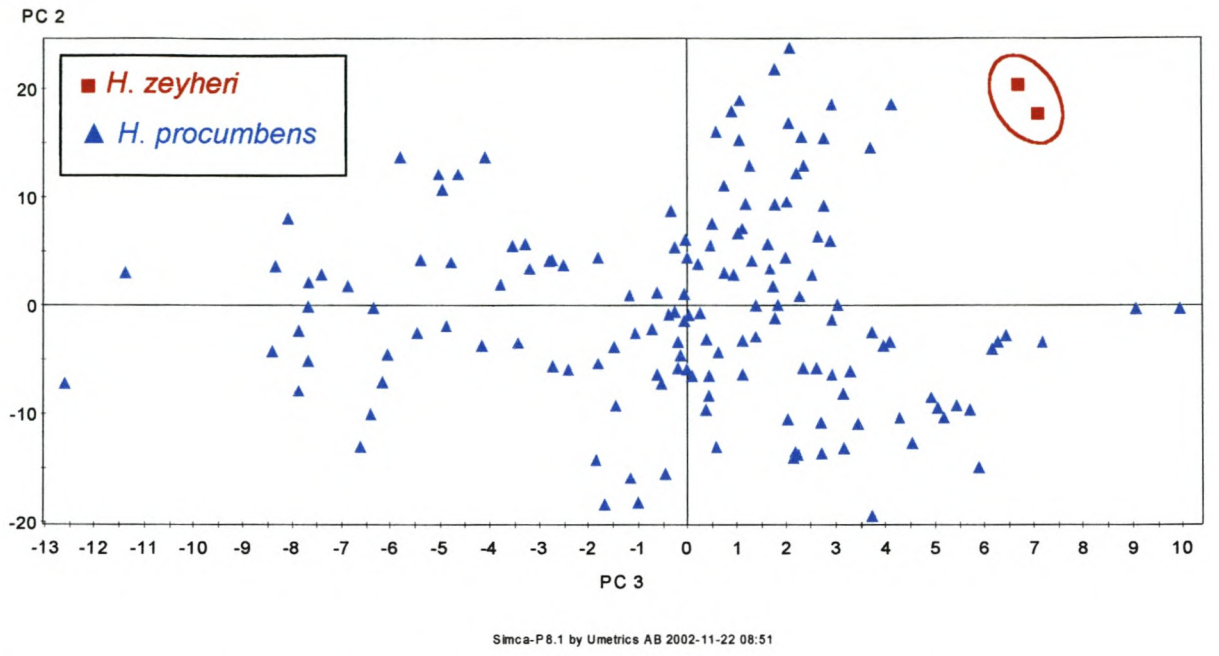


Figure 4.23 PCA plot from FT-NIR spectra of 150 dried, ground Devil's Claw samples indicating the potential classification of *Harpagophytum procumbens* and *H. zeyheri*. The second and third principle components are plotted against one another, since the first PC explains mostly particle size variation in these unprocessed spectra.

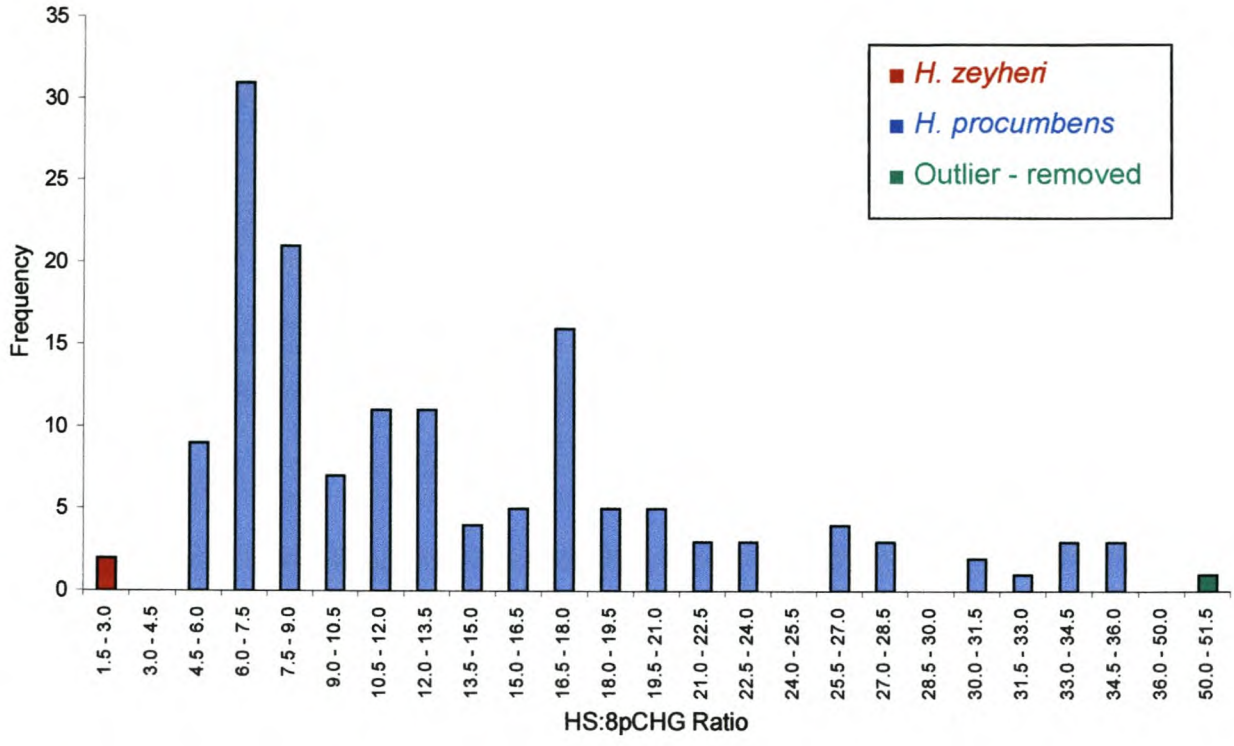


Figure 4.24 Histogram of the distribution of the harpagoside:8-ρ-coumaroyl harpagide (HS:8ρCHG) ratio (n = 150) of dried, ground Devil's Claw root. The two *Harpagophytum zeyheri* samples are indicated at the lower end of the range, with one outlier clearly visible at the maximum extreme.

Table 4.11 Summary of the predictive performance for harpagoside:8-ρ-coumaroyl harpagide (HS:8ρCHG) ratio using the FT-NIR and NIRSystems spectra of dried, ground Devil's Claw root, respectively. The *Harpagophytum zeyheri* samples have been removed from both calibrations, as well as selected outliers.

	HS:8ρCHG ratio Perkin-Elmer FT-NIR	**HS:8ρCHG ratio Foss NIRSystems 6500**
n	147	147
SEP	5.66	5.30
Bias	-0.09	0.77
r	0.603	0.610
PLS factors	5	5

n = total number of samples used (calibration and validation sets).
SEP = standard error of prediction (independent validation set).
r = correlation coefficient.
PLS factors = number of factors used to build the calibration model.

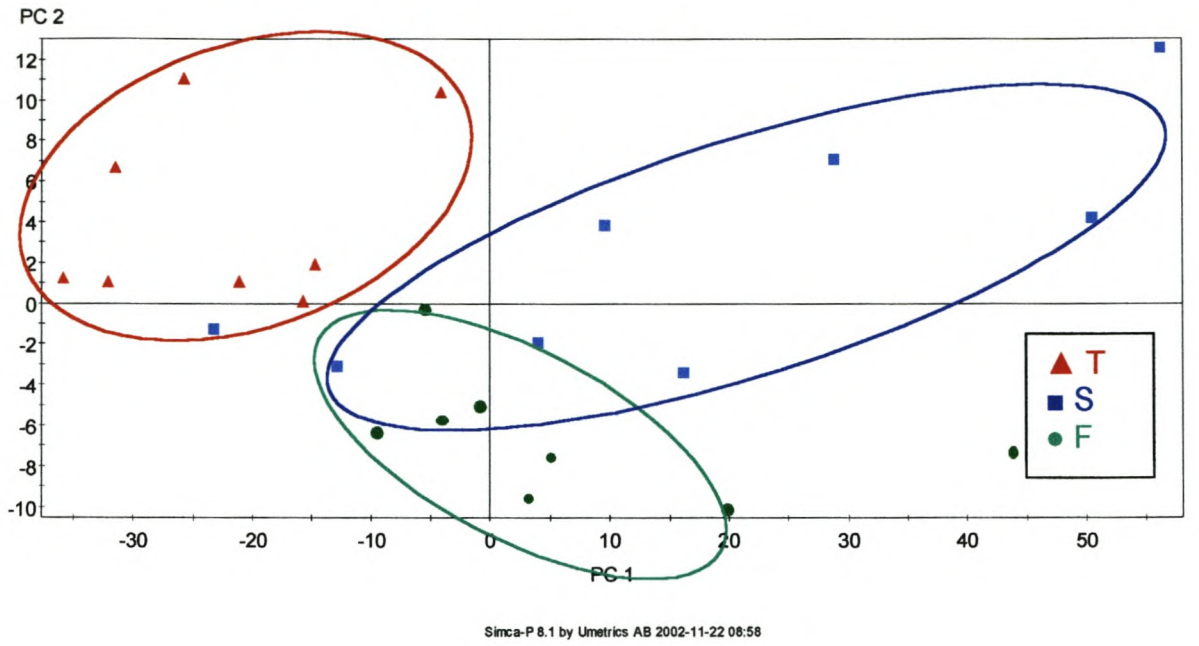


Figure 4.25 PCA plot (n = 24) of the spectra of Devil's Claw samples dried by three drying methods (T = tunnel-drying, S = sun-drying and F = freeze-drying).

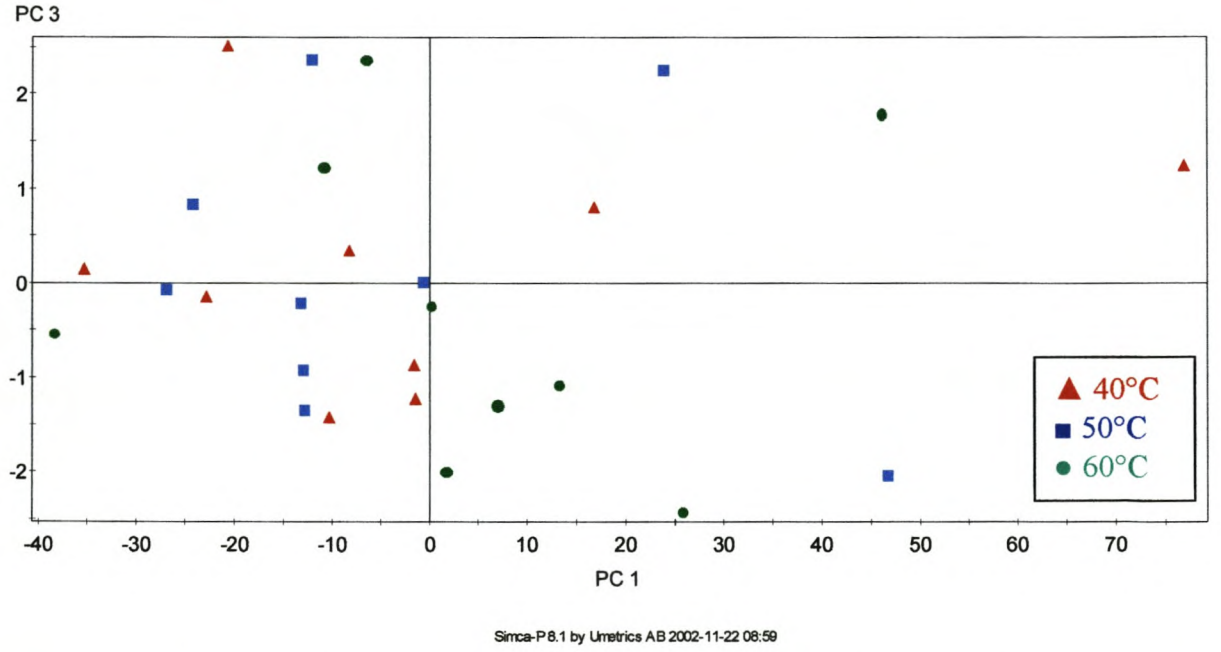


Figure 4.26 PCA plot (n = 27) of the spectra of tunnel-dried Devil's Claw samples dried at different temperatures.

correlates well with changes in MC and if the samples had been standardised to a similar MC value, this grouping would probably be less pronounced. It cannot be assumed, at this stage, that the grouping reflects the significant influence of drying method on HS retention.

Even though the statistical analysis of the results of the drying temperature study revealed significant differences between samples dried at 40°C and at 50°C, no clear groupings can be discerned in Figure 4.26. The MC range of the samples in this study was narrower (mean ± SD: 8.89% ± 1.10%) than the drying method study (4.89% ± 1.40%). This reflects the lack of evident groupings (Figure 4.26).

Conclusions

Moisture content of Devil's Claw samples could be successfully predicted by FT-NIRS analysis. From a quality control perspective, the rapid prediction of MC in dried Devil's Claw root is paramount to the storage stability of the product. Microbial spoilage that could possibly be detrimental to the harpagoside (HS) content, or could result in the formation of mycotoxins, would occur rapidly if the MC was too high. Prediction of the MC by NIRS methods would assist in ensuring the export quality of this indigenous product.

The results of this study also showed promise for the use of NIR technology as classification tool for the medicinal potency of Devil's Claw as linked to its HS content. Near infrared analysis may provide a rapid and easily used method to screen the root for HS classification purposes. The NIRSystems calibrations showed better predictive ability than the FT-NIR data, but neither of the two can rival HPLC as a quantitative method.

Based on the spectral variation in PCA plots, NIR also showed promise for the distinction between *Harpagophytum procumbens* and *H. zeyheri*. Although previous studies have used the ratio between HS and 8-ρ-coumaroyl harpagide (8ρCHG) as a possible means of differentiation, the present study found better separation between the species based solely on 8ρCHG values.

Further study is required to expand the calibration ranges for all the analytes, but especially for HS. This will assist in the development of more robust NIR models. The possibility of using a fibre-optic probe to scan samples should also be investigated. The direct contact between the probe and the sample would eliminate undesired scattering of light due to a glass vial or other container. Additionally, more *H. zeyheri* samples are required to confirm the separation of species by PCA.

It is believed that changes in the MC of samples during transport and storage led to inferior MC calibrations when using Foss NIRSystems 6500 spectra. The higher correlation and better predictive ability of the Foss spectra, in terms of analyte calibrations, are ascribed to an additional peak at *ca.* 2200 nm that correlates well with the chemical structure of the two iridoids

in question. Furthermore, although the MC of the samples might have changed, the applicability of the analyte reference values to the Foss spectra indicates stability of HS and 8ρCHG during transport and storage. Further investigation is needed to determine the maximum acceptable change in MC before the HS values are affected.

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Chapter 5

General discussion and conclusions

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Known from Khoi folklore, *Harpagophytum procumbens* (Devil's Claw) is an indigenous plant from the semi-arid regions of Southern Africa that is used for its medicinal properties, mostly linked to anti-inflammatory and analgesic activity (Ragusa *et al.*, 1984; Baghdikian *et al.*, 1997). The medicinal value is concentrated in the tuberous secondary roots of the plant (Czygan & Krüger, 1977, Franz *et al.*, 1982) with activity attributed to the glucoside iridoid, harpagoside (HS) (Guillerault *et al.*, 1994; Baghdikian *et al.*, 1997). Very little is, however, known concerning the stability of HS during the processing, transport or storage of the roots.

The ratio between HS and another iridoid, 8-ρ-coumaroyl harpagide (8ρCHG) may be used to distinguish *H. procumbens* from a second species, *H. zeyheri*, that is purported not to have medicinal effect (Baghdikian *et al.*, 1997; Eich *et al.*, 1998). This is of importance since Devil's Claw has an established economic value with approximately 600 tons exported per annum (Van Wyk & Gericke, 2000; Claassen, 2001). According to Chrubasik & Eisenberg (1999) suppliers of Devil's Claw are under no obligation to declare the iridoid content of their product. The potential for adulteration exists within this framework and there is a need to provide an accurate and rapid analysis technique that could assist in developing standardisation protocols. It has been suggested that the root be classified according to HS content into four classes: less than 1.2%, 1.2% to 1.6%, 1.6% to 2.5% and greater than 2.5% (G. Hansford, Grassroots Natural Products, Gouda, South Africa, personal communication, 2001).

Usually, high-performance liquid chromatography (HPLC) analyses have been used to determine the HS and 8ρCHG contents of the root (Guillerault *et al.*, 1994; Feistel & Gaedcke, 2000). This method is, however, lengthy, expensive and involves the use of large quantities of hazardous chemicals. As a rapid and non-destructive analysis technique, near infrared spectroscopy (NIRS) has proven useful in a variety of food related areas (Osborne *et al.*, 1993; Wetzel, 1998). It has also shown both quantitative and qualitative application in phytopharmaceutical products such as green tea (Schulz *et al.*, 1999), St. John's Wort (Huck, 2002) and ginseng (Ren & Chen, 1999).

This study evaluated the effect of processing conditions during the drying of Devil's Claw on the retention of HS. In particular, three drying methods (sun, tunnel and freeze-drying) and three tunnel-drying temperatures (40°C, 50°C and 60°C at 30% relative humidity) were assessed. Additionally, the possibility of using NIRS as an analytical tool for predicting the HS, 8ρCHG

and moisture contents (MC), and the HS:8ρCHG ratio of dried, ground Devil's Claw root was investigated.

The significant differences ($P < 0.05$) obtained in HS retention between sun-drying and the other two drying methods indicated that current practices using sun-drying do not produce the best possible product. This is especially true since typical sun-drying conditions may be even less favourable than they had been in this study. The observed trend, in order of HS retention, was freeze-drying > tunnel-drying > sun-drying. Visually, freeze-drying also proved the least detrimental drying technique, with preservation of both the colour and structure of the original roots. Economically, however, freeze-drying is not usually feasible and controlled tunnel-drying is recommended for the drying of Devil's Claw.

For tunnel-drying as method, it was shown that differences in HS retention can be attributed to the drying temperature used. Only the results of drying at 40°C and 50°C (dry bulb temperature) differed significantly ($P < 0.05$), but the observed trend suggested that drying at 50°C at a constant relative humidity (RH) of 30% would retain the highest HS content. There is, however, scope to further refine these conditions and research into optimising the tunnel-drying method is recommended. The economic implications for efficient tunnel operation should also be considered.

The decrease of HS content probably occurs through a variety of mechanisms, including enzymatic and non-enzymatic reactions, as well as oxidative and possibly polymerisation reactions. Drying under mild temperatures and high RH values for a long period of time, such as in sun-drying, favours enzymatic reactions (Lee-Kim *et al.*, 1995), while high temperature drying favours oxidative and other chemical reactions (Petrucci & Harwood, 1993). The enzymatic degradation of Devil's Claw can best be attributed to the action of polyphenol oxidase (PPO) (Vámos-Vigyázó, 1995) and β-glucosidase (Esen, 1993). The apparent browning of all tunnel and sun-dried samples can be ascribed to a combination of the action of PPO and non-enzymatic browning reactions, while β-glucosidase has been implicated in the hydrolysis of the glucosidic bond of iridoids. It is possible that the degree of browning, as observed in sun and tunnel-dried samples, is indicative of poor quality. A balance between drying temperature and time is therefore required to ensure both the quality of the product and cost-effectiveness of the process.

Two near infrared spectrophotometers were utilised to evaluate the feasibility of using NIRS as an analytical tool to determine HS, 8ρCHG and moisture contents, as well as the HS:8ρCHG ratio of dried, ground Devil's Claw root. Most of the calibrations were developed from the Perkin-Elmer FT-NIR Spectrum IdentiCheck spectra, but comparisons were made with models developed from Foss NIRSystems 6500 spectra. The individual performances of the analytes in question varied between the instruments, but both indicated good accuracy in

predicting HS and 8ρCHG contents. The slightly better performance of the Foss spectra can be attributed to an additional peak at *ca.* 2200 nm that correlated very well with the –C=O and –C=C– chemical bonds in both HS and 8ρCHG. Accurate MC calibrations were obtained from Perkin-Elmer spectra that could assist in predicting the stability of HS during export of the product. The observed discrepancy between the MC calibrations from the two instruments probably resulted from possible moisture uptake of the samples during transport to Germany for analysis on the Foss instrument.

The performance of the HPLC reference method was excellent and the NIRS models cannot compete in terms of quantitative measurements. The models did, however, show promise for application as screening methods in a possible HS grading system. It can therefore be concluded that NIRS can be used as a rapid and non-destructive qualitative analytical tool to determine HS content and thereby medicinal potency of dried, ground Devil's Claw root. Better understanding of the physico-chemical properties and changes within the Devil's Claw root is, however, required. Especially the chemical degradation of HS and the other gluco-iridoids, and the conditions required for such changes to occur, should be investigated.

Although the 8ρCHG models and PCA analyses of the spectra indicated possible use in determining species authenticity, further study with more *H. zeyheri* samples is needed for confirmation. Using the HS:8ρCHG ratio in this regard is recommended only as a supportive measure of authenticity, because it was shown to not be as sensitive as previously indicated in the literature.

In terms of the stability of HS, future research should concentrate on better understanding of the degradation mechanisms involved. This would allow for easier optimisation and maintenance of processing conditions. As mentioned above, improvement in NIRS models may be achieved by the inclusion of more *H. zeyheri* samples, but the HS range in *H. procumbens* samples should also be increased. This has implications for the cultivation of plants with inherently higher HS values. The feasibility of implementing a Devil's Claw classification system should also be investigated and NIRS has the potential to be an invaluable tool in this regard.

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Addendum A

Table A.1 Individual moisture and harpagoside (HS) contents of sun-dried samples for comparison of drying methods.

Sample Number	% Moisture	$\%HS_{as\ is}$*	$\%HS_{DB}$†
1	7.20	1.357	1.462
2	5.50	1.699	1.798
3	6.92	1.035	1.112
5	5.11	1.042	1.098
6	6.63	1.228	1.316
7	5.71	1.064	1.128
8	6.05	1.547	1.647
9	6.24	1.952	2.082
Average	6.17	1.366	1.455
Standard deviation	0.72	0.341	0.362

* $\%HS_{as\ is}$ = harpagoside concentration expressed on a 'wet mass' basis.
† $\%HS_{DB}$ = harpagoside concentration corrected for moisture (dry basis).

Table A.2 Individual moisture and harpagoside (HS) contents of tunnel-dried samples (60°C, 30% RH) for comparison of drying methods.

Sample Number	% Moisture	$\%HS_{as\ is}$*	$\%HS_{DB}$†
1	3.52	1.549	1.605
2	2.44	1.899	1.946
3	4.55	1.097	1.149
5	3.43	1.120	1.160
6	4.02	1.260	1.313
7	2.80	1.141	1.174
8	3.53	1.607	1.666
9	3.15	2.129	2.198
Average	3.43	1.475	1.526
Standard deviation	0.66	0.389	0.396

* $\%HS_{as\ is}$ = harpagoside concentration expressed on a 'wet mass' basis.
† $\%HS_{DB}$ = harpagoside concentration corrected for moisture (dry basis).

Table A.3 Individual moisture and harpagoside (HS) contents of freeze-dried samples for comparison of drying methods

Sample Number	% Moisture	$\%HS_{as\ is}$ *	$\%HS_{DB}$ †
1	6.32	1.554	1.659
2	5.06	1.926	2.029
3	5.06	1.136	1.196
5	4.35	1.119	1.169
6	4.93	1.283	1.350
7	3.37	1.168	1.209
8	4.84	1.650	1.734
9	6.70	2.024	2.170
Average	5.08	1.483	1.565
Standard deviation	1.05	0.362	0.394

* $\%HS_{as\ is}$ = harpagoside concentration expressed on a 'wet mass' basis.
† $\%HS_{DB}$ = harpagoside concentration corrected for moisture (dry basis).

Addendum B

Table B.1 Individual moisture and harpagoside (HS) contents of tunnel-dried samples (40°C, 30% RH) for comparison of drying temperatures

Sample Number	% Moisture	$\%HS_{as\ is}$*	$\%HS_{DB}$†
1	9.31	1.530	1.687
2	8.55	1.654	1.809
3	10.30	1.818	2.026
4	8.67	1.456	1.594
5	8.92	1.466	1.610
6	11.53	1.189	1.344
7	8.44	2.198	2.401
8	8.90	1.819	1.997
9	7.54	0.851	0.920
Average	9.13	1.553	1.710
Standard deviation	1.16	0.389	0.426

* $\%HS_{as\ is}$ = harpagoside concentration expressed on a 'wet mass' basis.
† $\%HS_{DB}$ = harpagoside concentration corrected for moisture (dry basis).

Table B.2 Individual moisture and harpagoside (HS) contents of tunnel-dried samples (50°C, 30% RH) for comparison of drying temperatures.

Sample Number	% Moisture	$\%HS_{as\ is}$*	$\%HS_{DB}$†
1	8.43	1.558	1.701
2	7.88	1.734	1.883
3	8.29	1.925	2.099
4	8.54	1.552	1.697
5	9.77	1.503	1.666
6	11.06	1.136	1.277
7	8.35	2.247	2.452
8	9.03	1.821	2.002
9	8.19	0.894	0.974
Average	8.88	1.597	1.750
Standard deviation	0.99	0.406	0.438

* $\%HS_{as\ is}$ = harpagoside concentration expressed on a 'wet mass' basis.
† $\%HS_{DB}$ = harpagoside concentration corrected for moisture (dry basis).

Table B.3 Individual moisture and harpagoside (HS) contents of tunnel-dried samples (60°C, 30% RH) for comparison of drying temperatures

Sample Number	% Moisture	$\%HS_{as\ is}$ *	$\%HS_{DB}$ †
1	8.86	1.590	1.745
2	8.33	1.710	1.866
3	7.73	1.930	2.092
4	9.10	1.475	1.622
5	9.60	1.381	1.527
6	11.05	1.103	1.240
7	8.60	2.213	2.421
8	8.41	1.850	2.019
9	6.70	0.908	0.973
Average	8.74	1.573	1.723
Standard deviation	1.39	0.410	0.444

* $\%HS_{as\ is}$ = harpagoside concentration expressed on a 'wet mass' basis.
† $\%HS_{DB}$ = harpagoside concentration corrected for moisture (dry basis).

Addendum C

Table C.1 Dilution series for 8-ρ-coumaroyl harpagide standard solutions (concentration of the stock solution = 0.97 $\mu g.\mu l^{-1}$).

Vol_{Stock} (μl)	Vol_{MeOH} (μl)	Total volume (μl)	$8\rho CHG_{mass}$ (μg)	Concentration ($\mu g.10\ \mu l^{-1}$)	Average area ± SD† ($\mu V.s$)
1	199	200	0.97	0.049	165017.5 ± 12127.6
1	149	150	0.97	0.065	182748.0 ± 3238.5
1	79	80	0.97	0.121	393722.5 ± 522.6
1	59	60	0.97	0.162	522092.5 ± 19589.0
1	49	50	0.97	0.194	596012.0 ± 21473.4
1	39	40	0.97	0.243	770505.0 ± 27077.9
1	29	30	0.97	0.323	977994.0 ± 93865.6
10	240	250	9.70	0.388	1206927.5 ± 1195.7
10	190	200	9.70	0.485	1452393.0 ± 65469.6
10	140	150	9.70	0.647	3985288.0 ± 71670.9
20	140	160	19.40	1.213	2135117.0 ± 15488.5
20	100	120	19.40	1.617	6192643.0 ± 193569.1
25	100	125	24.25	1.940	5180310.0 ± 210997.8
25	75	100	24.25	2.425	7967363.5 ± 337730.5

Vol_{stock} = volume (in μl) of the original stock solution used.
Vol_{MeOH} = volume (in μl) of HPLC-grade MeOH added.
$8\rho CHG_{mass}$ = mass of 8-ρ-Coumaroyl harpagide (in μg) in the final dilution (0.97 $\mu g.\mu l^{-1} \times Vol_{stock}$).
† Average area ± SD = average integration response (in $\mu V.s$) between duplicate HPLC injections ± standard deviation